# Innate Immune Responses to a Saponin Adjuvant in the Pig

## Application of Gene Expression Profiling

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# Innate Immune Response to a Saponin Adjuvant in the Pig. Application of Gene Expression Profiling


## Abstract

Vaccination is one of the most powerful ways to prevent infectious diseases. Successful vaccines produce a long-term immunity including effector T and B lymphocytes. In this context, adjuvants have a key role in vaccines by stimulating the innate immunity and thereby enhancing and shaping the subsequent adaptive immune response. The aim of this thesis was to elucidate the early innate immune response to the saponin-based adjuvant Matrix-M in the pig. Gene expression profiling was applied to monitor the global transcriptional response to Matrix-M in vivo. The innate immune response was further characterized by quantitative PCR analysis in vivo and in vitro and the early immunomodulatory effect of Matrix-M was evaluated in a contact exposure model. A mild inflammation and a cellular influx were recorded at the injection site and in the draining lymph node 24 hours after intramuscular injection of Matrix-M in pigs. In accordance, microarray analysis detected transcriptional alterations of genes for cytokines, chemokines and pattern recognition receptors in both tissues. Interferon-regulated genes were significantly overrepresented in these tissues, accompanied by increased gene expression for interferon-β in in the draining lymph node and interferon-α in blood. Transcriptional responses to Matrix-M in vitro were generally low but increased culture and exposure time affected genes for pro-inflammatory cytokines and $T_H$ cytokines in lymphocytes. Low levels of interferon-α gene expression were also detected in monocyte-derived dendritic cells. A contact exposure model was established to mimic field conditions when allocating grower pigs, by mixing pigs of various health statuses. After transport and mixing with conventionally reared pigs, all specific pathogen free (SPF) pigs in this model developed respiratory disease. Systemic symptoms that correlated with granulocyte counts, serum amyloid A levels and transcription of *IL18* and *TLR2* were provoked in two out of four SPF pigs that received saline prior to exposure, but not in those given Matrix-M. Taken together, the application of gene expression profiling successfully identified the induction of innate immune responses in porcine tissues and indicated that Matrix-M primes the host for further immune regulation. Thus, Matrix-M or similar saponin formulations are potentially useful clinical immunomodulators or adjuvants in emergency vaccines.



*Keywords:* porcine, adjuvant, ISCOM-Matrix, Matrix-M, microarray, qPCR, interferon, infection model.



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# Dedication

To my family.

*The truth is, most of us discover where we are headed when we arrive.*
Bill Watterson

# Contents

# List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Andersson, M., **Ahlberg, V.**, Jensen-Waern, M. & Fossum, C. (2011). Intestinal gene expression in pigs experimentally co-infected with PCV2 and PPV. *Veterinary Immunology and Immunopathology*, 142(1-2), pp. 72-80.

II **Ahlberg, V.**, Lövgren Bengtsson, K., Wallgren, P. & Fossum, C. (2012). Global transcriptional response to ISCOM-Matrix adjuvant at the site of administration and in the draining lymph node early after intramuscular injection in pigs. *Developmental and Comparative Immunology*, 38(1), pp. 17-26.

III Fossum, C., Hjertner, B., **Ahlberg, V.**, Charerntantanakul, W., McIntosh, K., Fuxler, L., Balagunaseelan, N., Wallgren, P. & Lövgren Bengtsson, K. (2014). Early inflammatory response to the saponin adjuvant Matrix-M in the pig. *Veterinary Immunology and Immunopathology*, 158(1-2), pp. 53-61.

IV **Ahlberg, V.**, Hjertner, B., Wallgren, P., Hellman, S., Lövgren Bengtsson, K. and Fossum, C. Transcriptional innate immune responses by Matrix-M adjuvant in pigs and its effects on natural infection. *In manuscript*.

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# Abbreviations

| | |
|---|---|
| AIM2 | Absent in melanoma 2 |
| CCL | (C-C motif) ligand |
| CD | Cluster of differentiation |
| cDNA | Complementary DNA |
| CpG | Cytidine-phosphate-guanosine oligodeoxynucleotides |
| $C_q$ | Quantification cycle |
| CTL | Cytotoxic T lymphocyte |
| CXCL | (C-X-C motif) ligand |
| DAMP | Damage-associated molecular pattern |
| DAVID | Database for annotation, visualization and integrated discovery |
| DC | Dendritic cell |
| DEG | Differentially expressed gene |
| FC | Fold change |
| GM-CSF | Granulocyte macrophage colony-stimulating factor |
| GO | Gene ontology |
| GSEA | Gene-set enrichment analysis |
| IFN | Interferon |
| IL | Interleukin |
| IRF | Interferon regulatory factor |
| IRG | Interferon-regulated gene |
| ISCOM | Immunostimulating complex |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |
| LPS | Lipopolysaccharide |
| MHC | Major histocompatibility complex |
| MoDC | Monocyte-derived dendritic cell |
| mRNA | Messenger ribonucleic acid |
| MyD88 | Myeloid differentiation primary response gene 88 |
| NET | Neutrophil extracellular trap |

| | |
|---|---|
| NLR | Nucleotide-binding oligomerization domain-like receptor |
| NLRP3 | NLR family pyrin domain containing 3 |
| NF-κB | Nuclear factor kappa-B |
| PAMP | Pathogen-associated molecular pattern |
| PBMC | Peripheral blood mononuclear cell |
| PCV2 | Porcine circovirus type 2 |
| pDC | Plasmacytoid dendritic cell |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| RIG | Retinoic acid-inducible gene |
| PMA | Phorbol 12-myristate 13-acetate |
| Poly I:C | Polyinosinic:polycytidylic acid |
| PPV | Porcine parvovirus |
| PRR | Pattern recognition receptor |
| qPCR | Quantitative real-time polymerase chain reaction |
| RNA-Seq | RNA sequencing |
| SPF | Specific pathogen free |
| STING | Stimulator of interferon genes |
| TGF | Transforming growth factor |
| $T_H$ | T helper |
| TLR | Toll-like receptor |
| TNF | Tumour necrosis factor |

# 1 Background

Vaccines have traditionally been constructed with the aim to defend the host against a pathogen by creating a protective and long-lasting immunity. Later, also therapeutic vaccines have been developed in order to re-direct or dampen immune responses, for example at allergic or autoimmune reactions, or to evoke and enhance immune reactions in immune-compromised individuals. For all these purposes it is necessary to understand how the immune reactivity can be modulated in a desired direction. Adjuvants have been defined as "components capable of enhancing and/or shaping antigen-specific immune responses" (Reed *et al.*, 2013) and is an important component in most vaccines. The word adjuvant is adapted from the Latin word *adjuvare*, meaning "to help". Their effects in combination with various antigen preparations have been scrutinized in several species, but effects of adjuvant components in the absence of antigen are less studied.

Saponins from the soapbark tree *Quillaja saponaria* Molina are effective adjuvants with immunomodulatory capacities. Purified fractions of such saponins in combination with cholesterol and phospholipids create nanoparticle adjuvant formulations known as ISCOM-Matrix or Matrix (Lovgren & Morein, 1988). Matrix formulations have successfully been used in a number of vaccines, but their mechanism of action in the absence of antigen is not fully understood. In the present thesis, transcriptomic methods were applied to elucidate early innate immune responses to a Matrix formulation in the pig. There is need for new and improved vaccines for pigs and the pig is an increasingly interesting model for human vaccine development (Dawson *et al.*, 2016; Fairbairn *et al.*, 2011).

## 1.1 Vaccine adjuvants

According to the "danger model", immune responses to antigen are elicited by danger molecules mainly detected by germ-line encoded pattern recognition receptors (PRRs; Matzinger, 2002). Danger signals can be derived from the host, as endogenous damage-associated molecular patterns (DAMPs; also called alarmins) or from pathogens, in the form of exogenous pathogen-associated molecular patterns (PAMPs). Triggering the innate immunity leads to production of cytokines that activate and modulate the ensuing adaptive immune responses to foreign antigens presented in that context.

Vaccines based on live, killed or attenuated microorganisms naturally contain PAMPs that activate the immune system, whereas purified proteins in subunit vaccines often have poor immunogenicity (Lövgren-Bengtsson *et al.*, 2016; Quinn *et al.*, 2013; Reed *et al.*, 2013). Most subunit vaccines thus need to be formulated with adjuvants in order to evoke long-lasting immune responses. Potent adjuvants are antigen sparing, reduce the need for booster doses and induce functional and cross-protective antibodies as well as cell-mediated immunity (Lee & Nguyen, 2015). Adjuvants exert these effects by promoting production and release of cytokines and chemokines that recruit immune cells to the local tissue, by antigen targeting to antigen-presenting cells that are activated and maturated and possibly also by facilitating antigen presentation (Awate *et al.*, 2013). Immune responses against vaccine antigens are typically divided into T helper ($T_H$) 1 types, effective against intracellular pathogens, and $T_H2$ types, effective against extracellular pathogens.

Adjuvants have been divided into three broad categories: immunomodulatory molecules, particulate formulations, and combinations of the two (Reed *et al.*, 2013; Cox & Coulter, 1997), as outlined in Table 1. Synthetically derived PAMP analogues, referred to as immunomodulatory molecules activate the innate immunity by triggering PRRs. Particulate formulations adsorb antigen and are thought to function as delivery systems to antigen-presenting cells (Reed *et al.*, 2013; Cox & Coulter, 1997), but convincing evidences for this concept still remains to be attained (Awate *et al.*, 2013). Several particulate adjuvants are however known to activate innate immune responses in an immunomodulatory manner, by up-regulation of cytokine genes and recruitment and maturation of immune cells (Caproni *et al.*, 2012; Mosca *et al.*, 2008; Seubert *et al.*, 2008).

The particulate formulation of aluminium salts, referred to as alum, has been the most widely used adjuvant in human vaccines since its discovery in the 1920s (reviewed in Marrack *et al.*, 2009). Alum enhances antibody production and induces $T_H2$ type immune responses, but gives no cytotoxic T lymphocytes (CTLs). Alum promotes immune responses in the absence of toll-

like receptor (TLR) signalling (Gavin *et al.*, 2006), but the NLR family pyrin domain containing 3 (NLRP3) inflammasome may be necessary for its adjuvant effect (Marrack *et al.*, 2009; Eisenbarth *et al.*, 2008). Similar to alum, all currently licenced adjuvants for human use are particulate formulations, or combined with one (Table 1). MF59 is a squalene oil-in-water emulsion used in influenza vaccines. It was originally described as a $T_H2$ adjuvant (Valensi *et al.*, 1994) although later studies suggest a more balanced $T_H1/T_H2$ response (Seubert *et al.*, 2008). The AS04 adjuvant has a non-toxic lipopolysaccharide-analogue combined with alum, which completely shifts the immune response from $T_H2$ to $T_H1$ (Didierlaurent *et al.*, 2009). Virus-like particles consist of virus envelope proteins without any genomic material, presenting antigen in a multimeric form that increase the immunogenicity (Morein *et al.*, 1978). Virus-like particles are taken up by antigen-presenting cells and the antigens are presented on both major histocompatibility complex (MHC) I and MHC II molecules (Kushnir *et al.*, 2012).

Based on the concept that engagement of PRRs activates the innate immunity, a number of specific PRR agonists have been evaluated as adjuvants, including the TLR agonists $Pam_3CSK_4$ (TLR2), polyinosinic:polycytidylic acid (poly I:C; TLR3), monophosphoryl lipid A (TLR4), flagellin (TLR5), imiquimod/resiquimod (TLR7/8) and CpG (TLR9). However, none of these have been licenced for human use (Table 1). Many of these molecules target receptor pathways for intracellular pathogen sensing, inducing type I interferons (IFNs) that can promote the induction of CTLs (Le Bon *et al.*, 2003). Another group of immunomodulatory substances is the saponins, which can also be formulated as a particulate adjuvant under certain conditions (Lovgren & Morein, 1988).

## 1.2 Saponin-based adjuvants

Adjuvant active saponins, particularly those extracted from *Quillaja saponaria* Molina, Quil-A. are potent immune modulators that have been used in animal vaccines for decades (Sun *et al.*, 2009). Traditional use of Quil-A in animal vaccines is in aqueous solutions but there are other formulations developed for increased activity or stability. Quil-A saponins have an inherent toxicity that springs from its affinity to cholesterol, thereby disrupting cell membranes and provoking subsequent lysis (Kensil *et al.*, 1991). A more recent development for use in human vaccines is the selected specific saponin compound QS21 (Kensil & Kammer, 1998). QS21 vaccines still have some tolerability issues and current clinical trials for QS21 are mainly intended for therapeutic vaccines (Bigaeva *et al.*, 2016), where tolerability is of less concern.

*Table 1.* Classification of selected adjuvants

| Adjuvant | Type of adjuvant | Type of immune response | Clinical status[a] |
|---|---|---|---|
| *Immunomodulatory molecules* | | | |
| $Pam_3CSK_4$ | TLR2 ligand | $T_H1$, $T_H2$, CTL | Preclinical |
| Poly I:C | TLR3 ligand | $T_H1$, CTL | Phase 2 |
| MPL (LPS analogue) | TLR4 ligand | $T_H1$ | Phase 3 |
| Flagellin | TLR5 ligand | $T_H1$, $T_H2$ | Phase 2 |
| Imiquimod | TLR7 ligand | $T_H1$, CTL | Phase 3 |
| Resiquimod | TLR7/8 ligand | $T_H1$, CTL | Phase 2 |
| CpG | TLR9 ligand | $T_H1$, CTL | Phase 3 |
| TDB | CLR ligand (Mincle) | $T_H1$, $T_H17$ | Phase 1 |
| QS21 | Saponin | $T_H1$, $T_H2$, CTL | Phase 3 |
| *Particulate formulations* | | | |
| Alum | Mineral salt | $T_H2$ | Licenced |
| MF59 | Oil-in-water emulsion | $T_H1$, $T_H2$ | Licenced |
| AS03 | Oil-in-water emulsion + α-tocopherol | $T_H1$, $T_H2$[b] | Licenced |
| Liposomes | Antigen delivery formulation | $T_H1$, $T_H2$, CTL | Preclinical |
| Virus-like particles | Antigen delivery formulation | $T_H1$, $T_H2$, CTL | Licensed[c] |
| *Combined formulations* | | | |
| AS01 | MPL + QS21 + liposome | $T_H1$, CTL | Phase 3 |
| AS02 | MPL + QS21 + AS03 emulsion | $T_H1$ | Phase 3 |
| AS04 | MPL + Alum | $T_H1$ | Licenced |
| GLA-SE | TLR4 ligand + emulsion | $T_H1$ | Phase 1 |
| ISCOM-Matrix | Matrix-formulated saponin | $T_H1$, $T_H2$, CTL | Phase 2 |

Modified after Reed *et al.* (2013), Awate *et al.* (2013), Lee and Nguyen (2015), Temizoz *et al.* (2016) and Apostolico *et al.* (2016). CpG, cytidine-phosphate-guanosine oligodeoxynucleotides; CLR, C-type lectin receptor; CTL, cytotoxic T cell responses; GLA-SE, glucopyranosyl lipid A stable emulsion; LPS, lipopolysaccharide; MPL, monophosphoryl lipid A; $Pam_3CSK_4$, tri-palmitoyl-S-glyceryl cysteine SK4; Poly I:C, polyinosinic-polycytidylic acid; TDB, trehalose-6,6-dibehenate; $T_H$, T helper; TLR, toll-like receptor.
[a] Status on clinical trials for human vaccines; [b] (Morel *et al.*, 2011); [c] (Kushnir *et al.*, 2012)

Purified fractions of Quil-A saponin are also used for formulation of immunostimulating complexes (ISCOMs.) The strong affinity between saponin and cholesterol is utilised to form the core matrix of the ISCOM, whereas phospholipids are needed for the inclusion of antigens (Lovgren & Morein, 1988). Binding of saponin to cholesterol also reduces the haemolytic and cytotoxic effects of the saponins.

### 1.2.1 ISCOM and Matrix formulations

The ISCOM is a cage-like 40 nm particle made from antigen, cholesterol, phospholipids and Quil-A (Morein *et al.*, 1984). It was formulated in an effort to combine the adjuvant activity of saponin in an immunogenic multimeric particle. The ISCOM generates superior antibody levels compared to antigen-containing micelles (Morein *et al.*, 1984). On top of improved antibody production, ISCOMs can induce MHC class I-restricted CTLs (Takahashi *et al.*, 1990). The immune response elicited by ISCOMs was protective at challenge with influenza virus in mice, both after mucosal and parenteral delivery (Lövgren *et al.*, 1990). In line with induction of CTLs, ISCOMs induce an immune response with both $T_H1$ and $T_H2$ cytokines (Sjolander *et al.*, 1997).

The original ISCOM formulation however suffered from some technical limitations. Not all types of antigen can be included in the ISCOM, the process of incorporating antigen is rather complex and the fixed antigen:saponin ratio is not always optimal as the amount of saponin needed for the ISCOM structure is often higher than what is needed for the adjuvant effect (Lövgren Bengtsson et al., 2011). However, ISCOMs without incorporated antigen, so-called ISCOM-Matrix or Matrix, work as an adjuvant when simply mixed with antigens. Matrix formulated with Quil-A saponins and cholesterol only, i.e. even without phospholipids, could increase the spontaneous proliferation of spleen cells collected from injected mice (Fossum et al., 1990). Matrix added to influenza virus micelles significantly increased antibody responses in vaccinated mice (Rönnberg et al., 1995) and Matrix mixed with influenza virus micelles elicited immune responses in mice with similar amplitude and characteristics as influenza virus ISCOMs did (Lövgren-Bengtsson & Sjolander, 1996). Matrix particles can also be formulated with fractions of Quil-A, designated QH-A, QH-B and QH-C (Rönnberg et al., 1995). Studies on QH-A and QH-C when included in ovalbumin ISCOMs indicated that QH-C is a more potent inducer of antibodies, whereas antigen-specific IFN-γ production is mainly dependent on QH-A (Johansson & Lövgren Bengtsson, 1999).

Matrix formulations based on purified saponin fractions have been commercialized as standalone adjuvants. ISCOMATRIX by CSL Ltd. is created from a mix of QH-A and QH-C at a 7:3 ratio (Morelli *et al.*, 2012). Isconova AB, acquired by Novavax Inc. in 2013, developed the formulations Matrix-Q from Quil-A and Matrix-M from a combination of Matrix-A and Matrix-C (formulations of QH-A and QH-C). By mixing the separately formed Matrix particles into Matrix-M, the dose of the more reactogenic Matrix-C can be reduced while maintaining the adjuvant effect (Lövgren Bengtsson *et al.*,

2011). Matrix-M and Matrix-Q were previously available as adjuvants for research purposes under the trade names AbISCO-100 and AbISCO-300, respectively.

### 1.2.2 Matrix-formulated saponin in vaccines

Similar to ISCOMs, antigen simply mixed with ISCOMATRIX induces strong antibody responses and CTLs (reviewed in Morelli *et al.*, 2012). In accordance, Matrix-M vaccines induce $T_H1$- and $T_H2$-related long-lasting antibody responses in mice and humans (Pedersen *et al.*, 2014; Magnusson *et al.*, 2013; Madhun *et al.*, 2009) and CTLs in mice (Quinn *et al.*, 2013). Multifunctional $T_H1$ $CD4^+$ cells producing interleukin (IL)-2, IL-12 and IFN-γ, typically considered to correlate with protection, are also induced by Matrix-M vaccines (Madhun *et al.*, 2009). Furthermore, both Matrix-M and ISCOMATRIX have an antigen dose-sparing effect in vaccines (Lövgren Bengtsson *et al.*, 2011; Maraskovsky *et al.*, 2009). Matrix-C was shown to promote immunity also in the presence of maternal antibodies (Heldens *et al.*, 2009). Matrix-C is used in a commercial horse influenza vaccine since 2006 (Equilis Prequenza Te; MSD Animal Health). Human clinical trials are currently ongoing for both Matrix-M[1] and ISCOMATRIX vaccines[2].

Matrix-M and ISCOMATRIX have been used together with TLR ligands in experimental vaccines. Matrix-M in combination with poly I:C increased the number of multifunctional $CD4^+$ T cells, and increased survival in experimental challenge to *Listeria monocytogenes* and vaccinia virus in mice (Quinn *et al.*, 2013). A vaccine adjuvanted with ISCOMATRIX in combination with both poly I:C and CpG was used therapeutically with some success against established tumours in a mouse melanoma model (Silva *et al.*, 2015). However, in a study on non-human primates, addition of CpG to a vaccine adjuvanted with Matrix-M did not enhance the antibody production, memory B-cell formation or the antigen specific $CD4^+$ response, speculatively due to sufficient $T_H1$ activation by Matrix-M alone (Martinez *et al.*, 2015).

The induction of CTLs by ISCOM-based vaccines is intriguing. In a study with Matrix-M, its ability to induce CTLs was similar to that of poly I:C, a strongly $T_H1$-prone adjuvant (Quinn et al., 2013). Induction of CTLs facilitated by ISCOMATRIX was required for the therapeutic effect against experimental melanoma in mice (Silva *et al.*, 2015). Antigen-specific CTLs requires MHC class I presentation, which in turn typically requires the antigen to be present in the cytosol as an intracellular pathogen or by means of cross-presentation by antigen-presenting cells. ISCOMATRIX can induce such cross-presentation in

1. http://novavax.com/page/11/clinical-stage-pipeline/
2. http://www.cslbehring.com/research-development/core-capabilities.htm/

conventional dendritic cells (DCs) by translocation of ingested antigen from the endosome/lysosome to the cytosol (Duewell *et al.*, 2011; Schnurr *et al.*, 2009). Maraskovsky *et al.* (2009) argue that this is not due to passive membrane disruption caused by saponins associating with cholesterol in cell membranes, as lysosomal acidification was required for the cytosolic translocation (Schnurr *et al.*, 2009). Furthermore, break of endosomal integrity correlated with inflammasome activation and pyroptosis in macrophages treated in vitro with ISCOMATRIX (Wilson *et al.*, 2014).

### 1.2.3 Cell migration and recruitment by Matrix-formulated saponin

Sheep lymph node cannulation experiments by Windon *et al.* (2000) showed that the total cell output from the local lymph node decreased immediately following injection with ISCOMATRIX. This phenomenon is referred to as "node shutdown" and is associated with an active immune response. The node shutdown remained for up to 48 hours, after which the output increased above baseline until six days after administration (Windon *et al.*, 2000). In the same study, neutrophils were recorded in the lymph after ISCOMATRIX administration. Rapid neutrophil infiltration after intraperitoneal injection in mice was described earlier also for ISCOMs containing influenza virus antigens (Watson *et al.*, 1989). In mice, neutrophils was the most increased cell type in both draining lymph node and spleen 48 hours after subcutaneous injection with Matrix-M (Reimer *et al.*, 2012). Also, the numbers of T and B cells, DCs ($CD11c^{+}$) and macrophages ($F4/80^{int}$) were increased in the draining lymph node and, to some extent, in the spleen. Dendritic cells showed increased expression of the co-stimulatory molecule CD86, indicating activation and maturation (Reimer *et al.*, 2012). Similar results were presented for ISCOMATRIX in mice, for which cell recruitment into the draining lymph node started already after 6 hours, and reached maximum 24 hours after administration (Wilson *et al.*, 2012; Duewell *et al.*, 2011). ISCOMATRIX administration in mice, using a subcutaneous air-pouch technique, also led to recruitment of neutrophils and monocytes to the administration site within 4 and 16 hours, respectively (Wilson *et al.*, 2012). The pronounced increases in cell recruitment and migration to the draining lymph node described above were not detected for alum, Freund's complete adjuvant or the squalene-based adjuvant AS03, in a comparative study with Matrix-M conducted in mice (Magnusson *et al.*, 2013). In contrast, Calabro *et al.* (2011) reported rapid recruitment of both neutrophils and monocytes to the injection site and draining lymph node after intramuscular injection both with alum and MF59. Neutrophils could not be detected in sheep after injection with soluble forms of

the saponins QH-A and QH-C (Windon *et al.*, 2000), suggesting that formulating saponins into Matrix is important for neutrophil recruitment.

### 1.2.4 Cytokine induction by Matrix-formulated saponin

Many studies on cytokine production induced by ISCOM-Matrix revolve around those produced by antigen-specific cells in recall experiments after vaccination. In both humans and mice, such studies show that Matrix-M induces $T_H1$ cytokines, such as IL-2 and IFN-γ, together with $T_H2$ cytokines, such as IL-4 and IL-10 (Pedersen *et al.*, 2014; Magnusson *et al.*, 2013; Madhun *et al.*, 2009). However, these results reflect the adaptive immune responses elicited by Matrix-M vaccines rather than the immunostimulatory effect of Matrix-M itself. The cytokine response in efferent lymph early after stimulation with ISCOMATRIX without antigen was studied in sheep by Windon *et al.* (2000). Within 24 hours the levels of CXCL8, IL-β, IL-6 and IFN-γ had increased, in contrast to IL-2 and tumour necrosis factor (TNF)-α. In mice, elevated serum levels of IL-6 and CCL4 were detected 48 hours after high-dose Matrix-M administration (Reimer *et al.*, 2012). Injection of ISCOMATRIX-like ovalbumin ISCOMs increased the levels of IL-1β, IL-5, IL-6 and IL-12p40 in the draining lymph node already after 6 hours compared to controls injected with phosphate-buffered saline or ovalbumin alone (Duewell *et al.*, 2011).

There are few reports on the direct effect of Matrix on cells in vitro. Wilson *et al.* (2012) noted that exposure of murine bone marrow-derived DCs and macrophages to ISCOMATRIX failed to induce any pro-inflammatory cytokines and that the bone marrow-derived DCs did not up-regulate any activation markers. In murine macrophages, Matrix formed with either Quil-A or a mix of QH-A and QH-C dampened the increase in IL-6 and TNF-α induced by inactivated respiratory syncytial virus (Hu *et al.*, 2001). However, macrophages primed with lipopolysaccharide (LPS) or TNF-α produced large amounts of active IL-1β and IL-18 after ISCOMATRIX stimulation in vitro (Wilson *et al.*, 2014). This induction was dependent on the NLRP3 inflammasome. Also Matrix-M and the soluble saponins Quil-A and QS-21 activate the inflammasome in vitro, after priming with TLR4 agonist (Marty-Roix *et al.*, 2016; Li *et al.*, 2008). In vivo, IL-18 was required for the production of IFN-γ by natural killer cells, induction of CTLs and production of $T_H1$ type antibodies by ISCOMATRIX vaccines (Wilson *et al.*, 2014). The in vivo IL-18 signalling was dependent on both myeloid differentiation primary response gene 88 (MyD88) and DCs, but unexpectedly not on NLRP3. Similar to in vivo-results for ISCOMATRIX, antigen-specific responses to a

QS-21 based vaccine were not reduced in mice lacking NLRP3 (Marty-Roix *et al.*, 2016).

Thus, there seems to be multiple pathways involved in the adjuvant activities caused by Matrix-formulated saponin. Some of the effects were shown also for free soluble *Quillaja* saponins, whereas some effects are attributed to the nanoparticle structure of the ISCOM-Matrix. Activation through NLRP3 could be detected in vitro after stimulation with Matrix, but was not crucial in vivo. In comparison, alum that signals through NLRP3 (Eisenbarth *et al.*, 2008) only induces $T_H2$-prone immune responses (Marrack *et al.*, 2009). Despite some insight into the early mechanisms, it is still open questions what processes that are necessary to cause the $T_H1$ responses typical for soluble and Matrix-formulated saponins.

### 1.2.5 ISCOM-based vaccines in the pig

Experimental and commercial ISCOM vaccines have been used in a number of species, including pigs, horses, cattle, sheep, dogs, cats, seals, chickens, mice and non-human primates (reviewed in Morein *et al.*, 2004). In the pig, early ISCOM vaccines against pseudorabies (Aujeszky's disease) demonstrated protection at lethal challenge on top of antibody and cellular immune responses (Tulman & Garmendia, 1994; Puentes *et al.*, 1993; Tsuda *et al.*, 1991). An ISCOM vaccine against *Toxoplasma gondii* conferred partial protection in pigs at challenge (Garcia *et al.*, 2005). Multiple trials have been conducted in gnotobiotic pigs with a rotavirus vaccine boosted with a combination of Matrix-formulated Quil-A and virus-like particles. Such vaccine formulation effectively increased the immune responses and reduced the symptoms at challenge both after oral (Nguyen *et al.*, 2003; Iosef *et al.*, 2002) and intranasal (Azevedo *et al.*, 2010; Nguyen *et al.*, 2006a; Nguyen *et al.*, 2006b; Gonzalez *et al.*, 2004) administration. An attenuated live vaccine against *Mycoplasma hyopneumoniae*, intended for intrapulmonary injection, induced high antibody levels and cellular immune responses after intramuscular injection when adjuvanted with Matrix-formulated Quil-A (Xiong *et al.*, 2014a). Compared to adjuvants based on carbomer, squalene or levamisole/chitosan, pigs receiving the vaccine with Matrix attained the highest cellular responses and had the lowest lung lesion scores after challenge (Xiong *et al.*, 2014b). Thus, the pig is effective for studying saponin-based vaccines, but Matrix formulations based on less toxic fractions of Quil-A have not yet been evaluated.

## 1.3 The porcine innate immune system in adjuvant research

Due to similarity in size, physiology, organ development and disease progression, the pig has been suggested as an ideal model for various human biomedical processes (Lunney, 2007). The pig is an outbred animal, but large-sized litters allow to some extent correction for individual variations by distributing littermates in the experimental groups (Lunney, 2007). According to Dawson, H. (as cited in Schook *et al.*, 2005), a comparison of the human, murine and porcine immune system revealed that more than 80% of the parameters analysed showed greatest similarities between pigs and humans, and less than 10% were more similar between mice and humans. A draft of the complete porcine genome (Ensembl build 10.2) was published in 2012 (Groenen *et al.*), showing that the evolutionary rate in the pig is similar to that in man and other mammals, except the mouse that has at least the double evolutionary rate. Consequently, the porcine genome is more similar to the human than the murine is. Of 500 immune genes analysed in man, mouse and pig, the mouse had 178 unique genes, versus 34 in pigs and 49 in man (Dawson *et al.*, 2013). Genomic similarity indicates functional similarity, and the main innate immune parameters likely to be affected by an immunomodulatory adjuvant will be discussed below for the pig.

### 1.3.1 Pattern recognition receptors

All major PRR families identified in human and mouse are present in the pig, including TLRs, NLRs, C-type lectin receptors, retinoic acid-inducible gene (RIG)-I-like receptors and absent in melanoma 2 (AIM2)-like receptors, with no large deviation in the number of genes compared to human (Dawson *et al.*, 2016). Overall, the PRRs identified are generally conserved between the pig and man. The importance of PRRs in porcine innate immunity is implied by the maintenance of a disproportionate amount of single-nucleotide polymorphisms located in the ligand-sensing parts of several porcine TLR genes, despite intensive breeding (Shinkai *et al.*, 2006). Using gene knockdown or overexpression, many porcine PRRs have been shown to respond to ligands known for human and murine PRRs, as detailed below.

TLRs are membrane-bound PRRs found on the cell surface (TLR1, -2, -4, -5, -6, -10) and in endolysosomal compartments (TLR3, -7, -8, -9). They can form homodimers (TLR3-5, TLR7-9), heterodimers (TLR2/TLR1 and TLR2/TLR6) or complexes with other molecules (TLR4 and e.g. CD14). All TLRs except TLR3 signal through MyD88 and nuclear factor kappa-B (NF-κB) to induce proinflammatory cytokines and this pathway is used also in pigs, as shown for TLR2 and TLR4 (Tohno *et al.*, 2007). Porcine TLR2 has ligands in common with its human counterpart (Alvarez *et al.*, 2008) and has a

similar tissue and cellular distribution, being expressed where the host is likely to meet pathogens, such as the skin, bronchial epithelia and lymphoid tissue (Alvarez *et al.*, 2008; Tohno *et al.*, 2006). Monocyte-derived macrophages up-regulate NF-κB-related genes after stimulation with the TLR4 agonist LPS (Kyrova *et al.*, 2014). Furthermore, TLR4, MyD88 and NF-κB were involved in up-regulation of *IL1B* after in vitro infection of porcine alveolar macrophages with porcine reproductive and respiratory syndrome virus (PRRSV; Bi *et al.*, 2014). Signalling via endosomal TLRs typically induce type I IFN after recognition of pathogen-derived nucleic acid; TLR3 through TIR-domain-containing adapter-inducing IFN-β (TRIF) and interferon regulatory factor (IRF)3/7 and TLR7 and TLR9 through MyD88 and IRF7. The requirement for these IRFs was confirmed for porcine TLR3 and TLR7 using poly I:C and imiquimod as agonists and gene overexpression in a human cell line (Sang *et al.*, 2008) and for TLR7 using gene knockdown in DCs (Alves *et al.*, 2007). Imiquimod is a TLR7-specific agonist in humans and mice but activates both porcine TLR7 and TLR8 (Zhu *et al.*, 2008). Porcine TLR9 is expressed in several lymphoid tissues and lack of expression was correlated with lack of responsiveness to CpG motifs (Dar *et al.*, 2008; Tohno *et al.*, 2006).

The RIG-I-like family consists of three cytosolic RNA sensors, including RIG-I itself. As for other species, porcine RIG-I was shown to signal through IRF3 and NF-κB, and RNA silencing of this receptor abolished the production of IFNα/β, IL-1β, IL-6 and TNF-α in porcine alveolar macrophages exposed to classical swine fever virus (Dong *et al.*, 2013). Several other RNA viruses also induced IFN-β gene expression through RIG-I (Hüsser *et al.*, 2011).

The NOD-like receptors (NLRs) are a family of cytosolic sensors for both DAMPs and PAMPs and triggering of these receptors can activate the inflammasome and/or signal through IRFs and the NF-κB pathway (Zhong *et al.*, 2013). Most known human NLRs were suggested from the porcine genome to be protein-coding genes also in the pig (Dawson *et al.*, 2016), including one of the most studied such receptor, NLRP3. Several substances known to promote NLRP3 inflammasome formation in other species, such as alum, ATP, calcium pyrophosphate dihydrate crystals and nigericin, also activated the porcine NLRP3 inflammasome (Kim *et al.*, 2014).

The C-type lectin receptor dectin-1 is present in pigs (Sonck *et al.*, 2009) and β-glucans trigger both dectin-1 and the complement receptor 3 but with some differences between cell types (Baert *et al.*, 2015). Detailed knowledge on ligands and signalling for other porcine C-type lectin receptors is however scarce (Mair *et al.*, 2014).

In the cytosol, DNA is detected by the inflammasome-forming AIM2-like receptors and stimulator of interferon genes (STING)-associated sensors that signal through IRF3 to trigger production of type I IFNs (Schlee & Hartmann, 2016). Only two porcine AIM2-like receptor genes have been identified, *MNDA* and *IFI16*, and although sharing names with the human AIM2-like receptors, they are not orthologous (Dawson *et al.*, 2016). The STING pathway has recently been described in other species as a central mediator for cytosolic DNA sensing that leads to type I IFN production (Schlee & Hartmann, 2016). In the pig, several cytosolic DNA sensors are expressed in various porcine tissues and were important for IFN-β induction by cytosolic DNA or pseudorabies virus in porcine cells (Wang *et al.*, 2015; Zhu *et al.*, 2014; Xie *et al.*, 2010). Only one of them was shown specifically to signal through STING in the pig, although all cytosolic DNA sensors require STING in humans. However, all porcine cytosolic DNA sensors described require IRF3 for type I IFN induction, and their presence show that the DNA sensing system is present and functional also in the pig.

Overall, many of the stimuli that activate innate immunity in other species can trigger porcine PRRs as well, and ligand specificities and down-stream signalling pathways generally seem to be comparable. Thus, the pig should respond to adjuvant formulations in a similar way as human and mouse.

### 1.3.2 Mononuclear phagocyte system

The mononuclear phagocyte system comprises some important innate immune cell types: monocytes, macrophages and DCs. These cells can, to a varying degree, initiate immune responses by production of cytokines and uptake of antigen for processing and presentation to lymphocytes. Categorization of cells belonging to the mononuclear phagocyte system has varied over time and been based on function, ontogeny or expression of cell markers, with no clear consensus (Vu Manh *et al.*, 2015; Guilliams *et al.*, 2014; Fairbairn *et al.*, 2011). Monocytes, macrophages and DCs constitute heterogeneous cell populations with a great plasticity and for many tissues there is no clear distinction between the populations, either in pigs, mice or humans (Vu Manh *et al.*, 2015; Fairbairn *et al.*, 2011; Summerfield & McCullough, 2009).

Porcine blood monocytes have been classified into two or four differentiation stages, depending on cell markers used (Fairbairn *et al.*, 2013; Chamorro *et al.*, 2005). In vitro, porcine monocytes may up-regulate MHC II and increase the transcription of IL-10, IL-12, IL-13 and IFN-γ in response to LPS, and they can also respond to CpG (Raymond & Wilkie, 2005).

Macrophages are tissue-resident cells that first encounter many of the pathogens. In vitro porcine macrophages can be generated with CSF-1 from

both monocytes and bone marrow, but their transcriptional responses to LPS differ prominently from those in alveolar macrophages (Kapetanovic *et al.*, 2013). In a study where porcine and murine bone marrow-derived macrophages were stimulated with LPS, the porcine responses were more similar to those of human monocyte-derived macrophages than the murine responses were (Kapetanovic *et al.*, 2012).

Dendritic cells are found both in blood and tissues and are roughly divided into conventional DCs and plasmacytoid DCs (pDCs). Plasmacytoid DCs are distinguished by their potent ability to produce IFN-α, whereas conventional DCs are typically defined as professional antigen-presenting cells. Porcine conventional DCs and pDCs are both present in blood with distinct phenotypes (Summerfield *et al.*, 2003). In tissues, the conventional DCs express somewhat different cell markers depending on the location, and may not always be separated from other immune cells (Mair *et al.*, 2014; Summerfield & McCullough, 2009). In porcine skin, four subsets of conventional DCs were described with various functional features (Marquet *et al.*, 2014).

To facilitate studies in porcine DCs, blood monocytes can be cultured with GM-CSF and IL-4 to generate monocyte-derived DCs (MoDCs; Johansson *et al.*, 2003; Carrasco *et al.*, 2001; Paillot *et al.*, 2001). Although ontogenetically different, MoDCs are phenotypically similar to conventional DCs in blood and tissues, and share many of their functional features. The role of IL-4 in pigs has been disputed (Raymond & Wilkie, 2005) and replacing IL-4 with IL-13 in combination with GM-CSF also produced porcine MoDCs (Bautista *et al.*, 2007). Furthermore, DCs can be generated in vitro from bone marrow with GM-CSF (Carrasco *et al.*, 2001) or with FMS-like tyrosine kinase-3 ligand (Guzylack-Piriou *et al.*, 2010). Porcine MoDCs express the genes for TLR2 (Alvarez *et al.*, 2008), TLR3 (Auray *et al.*, 2010) and TLR4 (Alvarez *et al.*, 2006) but low or no TRL5, -7 or -9 (Auray *et al.*, 2010; Alves *et al.*, 2007). Still, porcine MoDCs stimulated with LPS, poly I:C or imiquimod up-regulate the maturation markers CD80/86 (Auray *et al.*, 2010), and exposure to agonists for TLR2-5 can induce production of pro-inflammatory cytokines (IL-6, TNF-α), $T_H1$ cytokines (IL-12, IFN-γ) and/or $T_H2$ (IL-10, IL-13) cytokines (Auray *et al.*, 2010; Raymond & Wilkie, 2005).

Plasmacytoid DCs were originally identified as natural interferon-producing cells in porcine skin (Artursson *et al.*, 1995) and intestine (Riffault *et al.*, 2001), and associated lymphoid tissues, as well as in blood (Domeika *et al.*, 2004; Summerfield *et al.*, 2003). As in other species, porcine pDCs are cells that specifically respond to ligands for TLR7 and TLR9 by producing high levels of IFN-α, as well as IL-12 and TNF-α (Calzada-Nova *et al.*, 2010; Guzylack-Piriou *et al.*, 2004). Murine studies demonstrated that this is due to a

constitutive expression of IRF7 in pDCs (Honda *et al.*, 2005). Activation of pDCs has been related to neutrophils in the pathological condition systemic lupus erythematosus. In this autoimmune disease, neutrophils are primed to release neutrophil extracellular traps (NETs) containing cathelicidins and self-DNA that in turn activate pDCs through TLR9 (Garcia-Romo *et al.*, 2011; Lande *et al.*, 2011). Porcine neutrophils release NETs in a similar manner as human neutrophils (Brea *et al.*, 2012; Scapinello *et al.*, 2011) and porcine cathelicidins together with DNA have been shown to induce IFN-α production in pDCs (Baumann *et al.*, 2014).

Although no uniform subtyping of the cells belonging to the mononuclear phagocyte system exists, several lines of evidence suggest that there is no large discrepancy between the porcine system and those of other species. Furthermore, several in vitro systems are established that can be used to study immunomodulatory agents in the pig.

### 1.3.3 Interferons and interferon-regulated genes

The IFNs are a large family of cytokines with effects both on the innate and the adaptive immunity, especially regarding intracellular pathogens. Porcine IFNs consist of type I (IFN-α, IFN-β, IFN-δ, IFN-ε, IFN-κ, IFN-ω), type II (IFN-γ) and type III (IFN-λ) IFNs (Dawson *et al.*, 2013; Wang *et al.*, 2011). As in other species, porcine type I IFNs are known for their anti-viral properties. Type I IFN genes in pigs have undergone evolutionary expansion and contain two to three times as many genes as in human and mouse, including the eleven porcine-specific genes for IFN-δ (Dawson *et al.*, 2013). At least 39 type I IFN genes and 16 pseudogenes exist in the pig (Dawson *et al.*, 2013), with marked differences in gene expression between tissues (Sang *et al.*, 2010). A large number of the porcine type I IFNs display antiviral effect in vitro against PRRSV or vesicular stomatitis virus (Sang *et al.*, 2010) and IFN-α induction by poly I:C reduced the infectivity of PRRSV in alveolar macrophages (Miller *et al.*, 2009). Vector-induced expression of IFN-α can under experimental conditions protect pigs against infection with foot-and-mouth disease virus (Moraes *et al.*, 2003) and PRRSV (Brockmeier *et al.*, 2009).

Either type of IFN can induce an antiviral state in cells by inducing transcription of a large number of interferon-regulated genes (IRGs), including PRR genes (e.g. *OAS1*, *RIG-I*, most TLR genes) and those encoding IRFs and antiviral effectors (Schneider *et al.*, 2014). In this way, IRG induction primes the cell for further pathogen sensing and production of IFN and simultaneously limits the virus infection. IFN type I and III induce IRGs via the IFN-stimulated response elements (ISRE) promoter, whereas type II (IFN-γ) bind the gamma-activated sequence (GAS) promoter, resulting in different sets of

genes induced. The antiviral IRG products function either at the level of entry, replication or shedding of virus (Schneider *et al.*, 2014). Broadly acting antiviral genes identified for human cells include *IRF1*, *cGAS*, *RIG-I*, *MDA5* and *IFITM3* whereas other antiviral IRGs are either more or less virus-specific or exert their effect in combination with other IRGs (Schoggins *et al.*, 2011). The number of IRGs induced is typically mentioned to be in the range of hundreds (Schneider *et al.*, 2014; Schoggins *et al.*, 2011). However, the Interferome database[3] has to date identified over 3,000 IRGs each for type I and type II IFN by collecting data from high-throughput experiments of human and murine cells stimulated with IFN. Studies of this database show a large overlap of IRGs affected by type I and type II IFN.

Many IRGs were induced in blood after injection with poly I:C in pigs, with more genes being affected in pigs with high IFN-α levels (Liu *et al.*, 2014). Characterization of the numerous IRGs identified in various settings is often lacking, especially in the pig. However, the antiviral effect against PRRSV by type I IFN in porcine cells is to a great extent mediated through *MX1* (Sang *et al.*, 2010) and *OAS1* has an inhibitory effect on PRRSV infection in vitro (Zhao *et al.*, 2016). Studies using silencing RNA for *OAS1*, *CXCL10*, and *NRAMP1* showed that these genes were involved in antiviral effects for classical swine fever virus (Wang *et al.*, 2016). Induction of both IFN-α and IFN-γ in peripheral blood mononuclear cells (PBMCs) in vitro up-regulated the expression of *CXCL10* (Dar *et al.*, 2010), indicating that this gene is affected by both type I and type II IFN. But as IFN-α can stimulate porcine natural killer cells to produce IFN-γ (Toka *et al.*, 2009), it may also promote up-regulation of type II IFN-associated IRGs.

In conclusion, the main types of PRRs and cells that respond to defined immunomodulatory adjuvants are present in the pig, and resemble what is found in man. Recently, the pig was suggested as an intermediate species between mouse and man for immunological studies (Dawson *et al.*, 2016) and the pig should therefore be considered a suitable animal for adjuvant research.

## 1.4 Gene expression profiling

Immune cells respond to stimuli for example by secreting proteins or by changing their expression on or within the cell. Some of these proteins are preformed and released as full proteins or in processed forms. However, many proteins will require de novo synthesis after transcription of the corresponding gene. Several crucial events in immune cells thus occur at the level of transcription. Consequently, transcriptomics is a valuable tool alongside

3. http://www.interferome.org

proteomics and cellular profiling to examine host immune responses (Chaussabel *et al.*, 2010). Indeed, a large number of immune related genes are transcribed in the response to pathogens (Jenner & Young, 2005; Huang *et al.*, 2001). Many of these genes are shared for different stimuli, but individuals typically respond in a stimuli-specific and cell type-specific manner with defined kinetics (Jenner & Young, 2005; Huang *et al.*, 2001). Whereas quantitative real-time PCR (qPCR) remains the golden standard to measure transcription on a gene-to-gene basis, gene expression microarrays and RNA sequencing (RNA-Seq) allow for measuring the transcription of whole genomes without a pre-selection bias. This can be used to detect nuances of the responses to different pathogens or immunomodulatory agents. The first prototype microarray printed using robotics was a 48-probe array published in 1995 (Schena *et al.*). Microarrays are based on probes of oligonucleotides or complementary DNA (cDNA) to which complementary sequences in the sample can hybridize. Each probe or set of probes generally represents one gene and tens of thousands probes may be spotted per array. Current microarrays typically cover the whole genome. An alternative method for large-scale measurements of messenger RNA (mRNA) is the RNA-Seq technology, which allows an even higher resolution by detecting splice variants (Schroyen & Tuggle, 2015; Wang *et al.*, 2009).

Microarray is a semi-quantitative method, but is effective to identify gene expression alterations, i.e. differentially expressed genes (DEGs)(Malone & Oliver, 2011; Allison *et al.*, 2006). Gene expression profiling typically focus on identification of gene groups or functional pathways that are over-represented, "enriched", among these affected genes (Hedegaard 2009). With enrichment tools such as the Database for Annotation, Visualization and Integrated Discovery (DAVID)[4] and Gene-Set Enrichment Analysis (GSEA)[5], the gene expression of thousands of transcripts can be grouped based on functional annotations acquired from public databases, as Gene Ontology[6] (GO) or Kyoto Encyclopedia of Genes and Genomes (KEGG)[7]. Pathway analysis can be performed using for example Ingenuity Pathway Analysis (Qiagen) or InnateDB[8], which apply known protein-protein interactions to make connections between the DEGs. Furthermore, principal component analysis and cluster analysis can be used to find similarities in expression between samples and/or genes (Allison *et al.*, 2006).

4. https://david.ncifcrf.gov/
5. http://broadinstitute.org/gsea/
6. http://geneontology.org/
7. http://www.genome.jp/kegg/
8. http://www.innatedb.com/

### 1.4.1 Transcriptomic profiling of adjuvant effects

Host responses to pathogens and pathogen-derived molecules were explored by comparing a large number of already published microarray experiments on host-pathogen interactions (Jenner & Young, 2005). A common transcriptional response was identified and assigned to functional annotations such as (i) inflammatory cytokines, (ii) IRGs, (iii) transcription factors and signalling molecules that activate immune responses, (iv) anti-inflammatory factors, (v) lymphocyte activation, (vi) antigen presentation and (vii) cell adhesion (Jenner & Young, 2005). In a similar manner, a common set of "adjuvant core response" genes coding mainly for cytokines, chemokines and adhesion molecules were identified when analysing the global transcriptional response to the adjuvants alum, MF59 and CpG in injected mouse muscle (Mosca *et al.*, 2008). By revealing distinct differences in transcriptional responses to these adjuvants, the study emphasized that transcriptional profiling can provide insight into the mechanism of actions of adjuvants. Additionally, transcriptional changes could be related to increased protein expression in the tissue (Mosca *et al.*, 2008). Another study, comparing the global transcriptional responses to a $T_H1$-prone (monophosphoryl lipid A formulated in liposomes) and a $T_H2$-prone (alum) adjuvant in peritoneal exudate cells from intraperitoneally injected mice, supported the idea that early gene signatures are related to the subsequent type of adaptive immune response (Korsholm *et al.*, 2010).

The host responses detected may vary considerably depending on the tissue sampled. Later microarray studies on adjuvant effects have revealed large differences in genes induced between injected muscle, draining lymph node and blood from the same animal (Caproni *et al.*, 2012; Lambert *et al.*, 2012). Gene signatures of adjuvants detected in vivo may also differ from those observed in vitro (Caproni *et al.*, 2012). In humans, blood is the only easily accessible source for gene expression profiling of adjuvant responses in vivo. One way to overcome this limitation in mechanistic studies is to apply so called systems biology. Systems biology is the conceptual idea of combining traditional methods with emerging high-throughput methods in genomics, transcriptomics and proteomics for studies within the live animal, the whole "system" (Ideker *et al.*, 2001). Transcriptional responses can be successfully detected in human blood after administration of adjuvant (Caskey *et al.*, 2011) and together with antigen these gene expression signatures can be correlated with known immunological readouts (Pulendran *et al.*, 2010). This method was applied for the yellow fever vaccine YF-17D, for which early transcription of specific genes could predict subsequent induction of antibody levels (*TNFRS17*) and CTL responses (*GCN2*) (Querec *et al.*, 2009). This

computational procedure was later corroborated by studies in knockout mice revealing *GCN2* to be a key modulator of cross-presentation in DCs (Ravindran *et al.*, 2014).

Results obtained from systems biology studies in various species and settings (Obermoser *et al.*, 2013; Zak *et al.*, 2012; Nakaya *et al.*, 2011) support the notion that changes in gene expression are often associated with changes in protein expression and functional capacities. Consequently, this approach has recently also been used to find adjuvant-associated gene transcripts in blood that correlate with protection when combined with antigen (Nakaya *et al.*, 2016; Vaccari *et al.*, 2016). Taken together, transcriptional changes are clearly related to type of inducer, type of cell or tissue analysed, and time after exposure. This supports the use of transcriptomics as a useful method to decipher mechanisms of vaccine adjuvant in vivo.

### 1.4.2 Transcriptomic profiling of innate immune responses in the pig

In the pig, genome-wide transcriptomics emerged around 2003 when commercial microarray platforms became available (Tuggle *et al.*, 2007). The Qiagen NRSP8 porcine oligo array (designed in 2002) and the Affymetrix GeneChip porcine genome array (designed in 2004) were the first global arrays for pigs. Custom-made low-density spotted arrays containing less than 100 genes focused on porcine immune responses have also been developed (Andersson *et al.*, 2007; Ledger *et al.*, 2004). During the past few years, RNA-Seq has increased in popularity for porcine gene expression experiments, but the microarray technology is still more common (Fig. 1). The GeneChip array remains the most used microarray, but several genome-wide or immune-specific platforms are available today (reviewed by Schroyen & Tuggle, 2015). The porcine genome was published in 2012 (Groenen *et al.*), but a comprehensive annotation of immune-related genes was lacking until recently (Dawson *et al.*, 2016). As correct annotation is a hurdle in genome-wide transcriptomic studies in domestic animals (Hedegaard 2009), the full value of RNA-Seq for immunological studies has thereby been available for the pig.

At the initiation of this thesis in 2010, no global transcriptomic studies on adjuvants or vaccines in the pig were available. Early immunological studies using genome-wide microarrays in the pigs focused on response to infections, such as *Salmonella* Cholerasuis using the NRSP8 array (Zhao *et al.*, 2006) and studies on several pathogens using the GeneChip array: *Salmonella* Typhimurium (Wang *et al.*, 2007), *Salmonella* Cholerasuis (Wang *et al.*, 2008), *Haemophilus parasuis* (Chen *et al.*, 2009), classical swine fever virus (Durand *et al.*, 2009) and porcine circovirus type 2 (PCV2) (Lee *et al.*, 2010; Tomas *et al.*, 2010). These studies identified genes and pathways previously

not associated with the diseases and genes involved in resistance to disease, in some cases. Furthermore, the NRSP8 array expanded with swine leukocyte antigen complex (porcine MHC) genes had been validated using porcine PBMCs stimulated in vitro with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Gao *et al.*, 2010). Induction of cytokines and MHC genes recorded in that study could largely be confirmed by ELISA and flow cytometry.

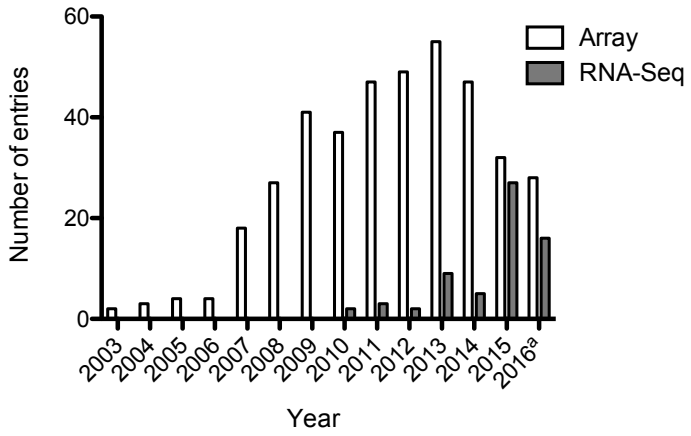


*Figure 1.* Porcine gene expression profiling studies over time. The graph displays number of deposited entries each year into the databases NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and ArrayExpress (https://www.ebi.ac.uk/arrayexpress/). Data acquired 2016-08-29. [a] Values for 2016 do not include the full year.

The transcriptomic studies performed up to that point in 2010 suggested that the tools for global gene expression profiling in the pig were readily available and effective and could be used to study responses to adjuvants in vivo. Furthermore, the pig has multiple features that make it a suitable study subject for adjuvant research. In contrast to many other animals typically used, the size of pigs allow multiple sampling of large blood volumes in the same animal, which can be used to simultaneously assess gene transcription, serum components and cell subsets. It is also possible to collect tissue samples from sites of administration and immunologically active organs in pigs, which is more restricted for human subjects. There are also vaccination routines and infection models in the pig that can be used experimentally to assess the effect of adjuvants alone or in combination with antigen. These advantages were exploited in the current thesis to explore the innate immune responses to the saponin-based Matrix-M adjuvant.

# 2 Aim and objectives

The aim of this thesis was to elucidate innate immune responses to the vaccine adjuvant Matrix-M in pigs, applying gene expression profiling.

The specific objectives were to:

- Establish methods for transcriptomic profiling of innate immune responses in porcine tissues
- Identify clinical, haematological and histological effects of Matrix-M administration in pigs
- Characterize the porcine transcriptional response to Matrix-M in vivo and in vitro
- Assess possible mode of actions of Matrix-M in innate immunity
- Evaluate prophylactic effects of Matrix-M in a porcine contact exposure model

# 3 Comments on material and methods

An overview of material and methods and considerations regarding some of the methods used are presented below. For detailed descriptions, see each individual paper (Paper I – IV).

## 3.1 Experimental designs

### 3.1.1 Pigs

To minimize unspecific activation of the immune system, effects of Matrix-M were studied exclusively in specific pathogen free (SPF) pigs and using blood cells collected from SPF pigs. Pigs aged nine to eleven weeks from two SPF herds were used. One of the SPF herds (Serogrisen; Ransta, Sweden) originated from caesarean-derived colostrum-deprived piglets (Wallgren *et al.*, 1999). Pigs from this herd were used in the two in vivo experiments with Matrix-M, performed at the animal facility of the National veterinary institute (Uppsala, Sweden; Paper II, IV). The other SPF herd (Swedish Livestock Research Centre; Lövsta-Uppsala, Sweden) was established in 2012 from the first SPF-herd, and these pigs were used for the in vitro studies with Matrix-M (Paper III, IV). The SPF herds were declared free from most major swine pathogens (Wallgren *et al.*, 1999) but were known to harbour PCV2. Maternal antibodies to *Haemophilus parasuis* were found in the SPF herd used for the in vivo experiments (Paper IV). Both PCV2 and *H. parasuis* may induce disease in the presence of environmental stressors, but no clinical signs of disease associated with PCV2 or *H. parasuis* were present in the SPF herds.

Conventionally reared pigs were used for a contact exposure model (Paper IV). The health status of pigs in Sweden is generally high, but common pathogens in conventional pig farms causing respiratory or systemic disease include *Actinobacillus pleuropneumoniae*, *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Streptococcus suis* and *H. parasuis*. PCV2 is present in

most herds, but herds that have experienced PCV2-associated diseases are generally vaccinated against PCV2. PRRSV has not been reported in Sweden since 2007[9]. All of the listed pathogens are involved in the so-called "porcine respiratory disease complex" that may interact with stressors from environment and management to induce disease in for example grower pigs (Opriessnig *et al.*, 2011). The conventionally reared pigs used in Paper IV originated from a farrow-to-finish herd with a high prevalence of respiratory lesions recorded at slaughter. These pigs had typically high levels of serum antibodies to *A. pleuropneumoniae* and to *P. multocida* at 19 weeks of age and PCV2 was known to be present in the herd, although without clinical signs of PCV2-associated disease. Thus, it was assumed that any SPF pigs mixed with these pigs would develop respiratory and/or systemic disease with time.

### 3.1.2 Administration of Matrix M and contact exposure model

All experiments were carried out with Matrix-M (AbISCO-100) except in the tolerability study in which Matrix-Q (AbISCO-300) was used. Matrix-Q was administered subcutaneously in three dosage options (75 μg, 100 μg, 150 μg; Paper III) whereas 150 μg of Matrix-M was injected intramuscularly (Paper II, IV). Doses were based on published data and previous experience from other species. In comparison, the Matrix-M dose used in human clinical trials is 50 μg (Cox *et al.*, 2011). The adjuvants were obtained from Isconova AB that is currently acquired by Novavax Inc. Matrix-M was suspended in saline to minimize irritation from the vehicle and was administered intramuscularly into the thigh to be able to locate the injection site and the draining lymph node(s) (Paper II, IV). Matrix-Q was given subcutaneously to better be able to assess the local reaction (Paper III).

The early local immune response to Matrix-M (Paper II) was evaluated in SPF pigs exposed to as few stressors as possible. The pigs were allocated into groups at the farm of origin 14 days before delivery and given a 48-hour acclimatization period after the transport to the animal facility before administration of Matrix-M or saline. The pigs were sacrificed 24 hours later because mice injected with various adjuvants had the greatest number of genes up-regulated in muscle after 24 hours (Mosca *et al.*, 2008). Also sheep administered ISCOMATRIX displayed maximum lymph node reaction 24 hours after injection coinciding with maximum cytokine output at this time (Windon *et al.*, 2000). Thus, 24 hours after injection of Matrix-M was chosen as an appropriate time point for detection of changes both at the injection site and in the draining lymph node.

9. http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/Diseasedistributionmap/

In Paper IV, the effect of Matrix-M on stress induced by transport and mixing coupled with contact exposure to pigs with different health status was evaluated. In order to mimic field conditions experimentally, SPF pigs were co-mingled with conventionally reared pig or with non-littermate SPF-pigs. No acclimatization period was applied and these SPF pigs were administered Matrix-M at the farm of origin the day before transport to the animal facility. Four hours after arrival, the SPF pigs were mixed with conventionally reared pigs. The experiment was designed to follow the transcriptional response to Matrix-M in blood but was adapted in length with the aim to still have active transcriptional responses to Matrix-M in the local tissues at the termination. Thus, all pigs were sacrificed six days after injections.

### 3.1.3 Evaluation of adjuvant reaction and disease parameters

The general condition of the pigs and adverse reaction at the injection site was assessed in all in vivo experiments (Paper II, III, IV). At post-mortem examination, the muscle at the injection site and its draining iliac lymph node was specifically examined for macroscopical alterations to detect reactions to Matrix-M (Paper II, IV). As the SPF pigs in Paper IV were assumed to develop illness due to the contact exposure to conventionally reared pigs differential WBC and serum levels of SAA were used to follow the disease progression in blood (Cray *et al.*, 2009; Hulten *et al.*, 2003). Respiratory signs were recorded for these pigs using a scale from 0 to 3 and criteria previously applied in experimental infection with *A. pleuropneumoniae* (Sjolund *et al.*, 2009). Post mortem, lesions in bronchial lymph nodes, lung and joints were recorded as indications of infectious disease (Paper IV).

### 3.1.4 Tissue sampling and histological evaluation

Tissue samples from the muscle at injection sites and internal iliac lymph nodes were collected immediately after death (Paper II, IV). Samples were transferred to RNAlater and kept overnight at 4°C followed by long-term storage at -70°C (according to manufacturer's directions), snap-frozen in dry ice-cooled isopentane before immediate long-term storage in liquid nitrogen, or fixed in formalin. RNAlater allow quick stabilization of the RNA in the tissue and storage at ambient temperatures for limited periods, at least a week according to the manufacturer. RNAlater is also useful to limit degradation when thawing the samples for RNA extraction. Formalin-fixed samples were embedded in paraffin and sections were stained with haematoxylin and eosin for histological evaluation, which was done in collaboration with a senior veterinary pathologist (Paper II). Thus, precautions were taken to limit the destruction of RNA and to preserve samples for putative future analysis.

## 3.2 In vitro exposure to Matrix-M

### 3.2.1 Stimulation of cell cultures for gene expression analysis

PBMCs that are commonly used to study immune reactivity in vitro were used to measure transcriptional responses to Matrix-M (Paper III and IV). As not all cells are likely to take part in the response, or respond in a similar fashion, subpopulations of PBMCs were established by in vitro depletion, enrichment or differentiation. Because DCs are critical for a strong adaptive immune response and may be targeted by vaccine adjuvants (Liang & Lore, 2016), MoDCs were generated for exposure to Matrix-M. Monocytes were also used for Matrix-M exposure studies.

Blood collected from SPF pigs in heparinized tubes, to reduce clotting, was processed within one hour to reduce non-specific activation of cells (Paper III, IV). PBMCs were isolated by centrifugation on Ficoll-Paque PLUS and monocytes were isolated from PBMCs by plastic adherence. MoDCs were generated by culturing monocytes for five days in the presence of rpIL-4 and rpGM-SCF, as previously described (Johansson *et al.*, 2003; Carrasco *et al.*, 2001; Paillot *et al.*, 2001). MoDCs generated in this way express several TLRs and can be induced to produce both pro-inflammatory cytokines and IFN-α (Auray *et al.*, 2010; Johansson *et al.*, 2003), and they are efficient at both receptor- and non-receptor-mediated endocytosis (Paillot *et al.*, 2001). Plastic adherence of human monocytes for generation of MoDCs has been reported to affect their cytokine expression (Elkord *et al.*, 2005), but this effect was not detected for porcine MoDCs (Auray *et al.*, 2010).

Cell cultures were exposed to Matrix-M, LPS or ODN 2216 (Paper III, IV). Short-term 6-hour exposures were made for freshly isolated PBMCs (Paper III), monocytes cultured overnight, lymphocytes cultured overnight or for three days and MoDC generated for five days (Paper IV). The transcriptional host response to immunomodulatory agents typically follows a temporal pattern (Jenner & Young, 2005), and gene transcripts induced by Matrix-M may escape detection at a short-term exposure. Long-term exposures were therefore made for lymphocytes and MoDCs. This allowed the cells to respond not only to Matrix-M directly, but also to molecules promoted initially by Matrix-M and possibly to DAMPs released from the cells.

### 3.2.2 Induction of neutrophil extracellular traps

Isolation of porcine polymorphonuclear leukocytes was made after removal of erythrocytes from blood by dextran sulphate by using a discontinuous gradient of 70% and 80% Percoll (Paper III; Dom *et al.*, 1992). The pre-removal of erythrocytes was essential since a gradient alone will not certify a complete

separation between polymorphonuclear leukocytes and erythrocytes in porcine blood (Roberts *et al.*, 1987). Polymorphonuclear leukocytes experiments were performed in serum-free media, since nucleases that degrade NETs may be present in serum (von Kockritz-Blickwede *et al.*, 2009). Instead, 2% BSA was used to facilitate adherence of neutrophils (Brinkmann *et al.*, 2010). The best known inducer of NETs is PMA and the concentration used in the current thesis has been described to produce NET-like structures from porcine neutrophils (Scapinello *et al.*, 2011). Matrix-M was tested in various concentrations: 0.3, 1 and 3 μg/ml. After stimulation for four hours, culture media was removed and DNA was visualized by addition of SYTOX Green for 10 min followed by fixation with formaldehyde for 30 min, both in dark. Although SYTOX Green is non-permeable, this method allowed for staining of both extracellular and intracellular DNA with SYTOX without disrupting the sensitive NET-like structures.

## 3.3 Gene expression analysis

Microarray technology was used to measure the global transcriptional response to Matrix-M in muscle and draining lymph node, providing an unbiased gene expression profile (Paper II). Prior to this, the same microarray was applied to intestinal tissue obtained from pigs experimentally infected with PCV2 and porcine parvovirus (PPV) in order to consider the method and gain experience in evaluating the data generated (Paper I). The microarray analyses were complemented with qPCR analysis in these two studies. The transcriptional response in blood to Matrix-M administration was screened with a qPCR plate array, and selected up-regulated genes were confirmed by single qPCR assays (Paper IV). Expression in cell cultures exposed to Matrix-M or other inducers was also measured with qPCR (Paper III, IV).

### 3.3.1 RNA isolation

RNA from cell cultures and all tissues except blood was extracted using a combination of Trizol reagent and RNA purification spin columns (Paper I, II, III, IV) described by Wikström *et al.* (2011). This method avoids the use of homogenization columns, reduces the risk of contamination from Trizol, and allows DNA to be acquired from the same samples (Paper I). Muscle samples were homogenized in Trizol by a stator-rotor homogenizer and samples from intestine and draining lymph nodes were homogenized in Trizol using a mechanized pestle and passing through an 18G needle multiple times. The pestle disrupted muscle and connective tissue to a low degree, which enriched samples for RNA from immune cells. Cell cultures were homogenized by

repeatedly pipetting the Trizol mixture up and down. All cell and tissue homogenates were brought up in 1 ml Trizol to make the downstream protocol equal regardless of source. After phase separation, the RNA-containing aqueous phase was loaded onto E.Z.N.A Total RNA Kit columns for purification of the RNA.

PAXgene Blood RNA Tubes that immediately stabilize RNA were used to collect whole blood for gene expression analysis (Paper IV), and the corresponding PAXgene Blood RNA Kit was used for RNA isolation. These tubes are designed for human use and proved somewhat difficult to use in pigs. The amount of blood collected in these tubes is normally limited and did also not always fill up completely, leading to low RNA yield for some samples. These RNA samples were further concentrated by the E.Z.N.A. MicroElute RNA Clean Up Kit.

### 3.3.2 RNA quality control

Quantity (260 nm) and purity (260/230 and 260/280) of isolated RNA was determined by absorbance using a Nanodrop spectrophotometer. Quite a few samples were below the recommended ratio of 1.8 for 260/230, especially RNA samples from in vitro experiments or those with low yield. No correlation was found between low 260/230 ratios and qPCR efficiency, as estimated both by LinRegPCR[10] and expression analysis of reference genes. As possible contaminants not seemed to affect the qPCR reaction, no cut-off was used based on purity as assessed by the absorbance values.

The quality of RNA from in vivo samples was measured by the capillary gel electrophoresis systems Bioanalyzer (Paper I, II) and Experion (Paper IV), which score the RNA integrity on a scale from 1 to 10 as an RNA Integrity Number (RIN) or an RNA Quality Indicator (RQI), where intact RNA have RIN > 8 (Fleige & Pfaffl, 2006). RIN and RQI values are calculated slightly different but give comparable results (Pfaffl *et al.*, 2008). The quality of all samples for microarray analysis (Paper I, II) was analyzed, but was not routinely performed for all other samples. RNA samples from intestine from the PCV2-infected pigs (Paper I) displayed large variation in integrity and the three samples from each group with the highest RIN were selected for microarray analysis (RIN 6.2 – 7.8). Average RIN values reported from bovine intestines ranged from 4.6 to 7.5 (Fleige & Pfaffl, 2006). RNA from the Matrix-M in vivo experiment (Paper II) was extracted from tissue samples stored in RNAlater and displayed RIN values from injection site ranging from 7.4 – 9.4 and from draining lymph node ranging from 7.2 – 9.5. A representative selection of blood RNA samples was evaluated in Paper IV,

10. http://www.linregpcr.nl/

indicating a high quality (RQI > 8) of the RNA isolated and purified with the applied method.

### 3.3.3 Synthesis of cDNA

Due to various numbers of cells in the cell cultures and varying amount of tissue prepared, different amounts of RNA was used for the synthesis of cDNA. Equal amounts of RNA were used within each experiment to facilitate comparisons between treated and untreated samples. Despite separation of RNA from DNA using Trizol or on-column DNase treatment with the PAXgene kit, contamination with genomic DNA was still possible. Intron-spanning qPCR assays are unaffected by genomic DNA contamination, but many genes do not allow such primer design. Thus, genomic DNA had to be kept at a minimum. RNA samples isolated using Trizol were treated with RNase-free DNase (Promega) before cDNA synthesis, but PAXgene-isolated RNA was degraded during the heat-inactivation step. The DNA-free DNA Removal Kit that applies protein precipitation for DNase inactivation was therefore used for the PAXgene RNA.

First strand cDNA was synthesised using Superscript II Reverse Transcriptase (Paper I, II, III;) or the GoScript Reverse Transcription System (Paper III, IV). To confirm the removal of genomic DNA, an intron-less IFN-α qPCR assay was performed on non-reverse transcribed control samples (Trizol samples) or on the DNased RNA (PAXgene samples).

### 3.3.4 Reverse transcription qPCR

In Paper I, II and the in vitro part of Paper III, qPCR assays based on hydrolysis probes (TaqMan) previously established in the lab were used for gene expression analysis (Wikström *et al.*, 2011; Timmusk *et al.*, 2009). In Paper IV and the in vivo part of Paper III, SYBR Green qPCR assays were used. Assays based on hydrolysis probes may be more specific than SYBR Green, but lack the possibility for melt-curve analysis. SYBR Green binds any DNA and can cause accidental false positives due to non-specific amplicons or primer-dimers, but analysing the melt curve can identify these. As long as both methods are specific, they provide equal estimation of gene expression (Arikawa *et al.*, 2008). Non-specific products for the SYBR Green qPCR assay were rare in the optimized assays in the current thesis, except for late appearing (high $C_q$) products in no-template control reactions. Primer pairs for SYBR Green qPCR were taken from the hydrolysis probe assays (IFN-α, IFN-γ, *IL1B*, *IL6*, *IL10*, *IL12B*, *TNF*, *TGFB1*), from published works (*GAPDH*, *HPRT*, IFN-β, *PPIA*, *RPL32*, *SPP1*, *STING*, *TLR2*, *TLR4*, *YWHAZ*) or were designed in house (*CXCL8*, *IFITM3*, *IL17A*). All primer pairs were optimized

or re-optimized for the current SYBR Green qPCR kit and qPCR platform (Table 1 in Paper III, Table 1 in Paper IV). A custom SYBR Green qPCR plate array containing 92 innate immunity genes was used for screening of gene expression in blood after Matrix-M administration (Paper IV). Genes on the array were selected based on general knowledge on innate immune responses as well as genes indicated from the microarray study, and included for example genes for interleukins, IFNs, IRGs, chemokines and chemokine receptors, PRRs and associated adaptor proteins and transcription factors. Due to the number of total samples (8 pigs, 6 time points), pooled RNA from each group was used for discovery of possible DEGs, which were selected for further validation. A number of genes (*IL18*, *MYD88*, *NLRP3*, *TLR4* and *TLR9*) from the plate were analysed on individual samples using the same commercial assay in single assay format.

All hydrolysis probe assays were run in triplicates, whereas SYBR Green assays were run in duplicates. Triplicates allow for removing outliers but duplicates need to be reanalysed in case of diverging results. However, the variability between replicates was typically low, which supported the use of only duplicates. Melt curve analysis was performed after each SYBR Green qPCR run. Limit of detection was not established despite being recommended in the MIQE guidelines (Bustin *et al.*, 2009). For the SYBR Green assays, samples were regarded as not detected or non-quantifiable if $C_q > 35$ for either replicate or if there were large differences between replicates for samples with high $C_q$ values.

### 3.3.5 Reference genes and normalisation of gene expression

Differences in RNA quality, RNA amount in the cDNA synthesis and effectiveness of the cDNA synthesis reaction will affect the threshold cycle values obtained for all genes analysed in a sample. Normalising $C_q$ values for genes of interest against the expression of reference genes can correct for this. Reference genes are selected among genes that have stable expression despite experimental treatment. Using several reference genes reduce the risk of errors, especially when detecting small differences in expression (Vandesompele *et al.*, 2002). As this was anticipated for gene expression in blood (Paper IV), a number of reference gene candidates were tested using the geNorm algorithm in the software qBase$^+$. The algorithm uses a pair-wise exclusion approach to select the most stably expressed genes in a data set. The genes *GAPDH*, *HPRT*, *PPIA*, *RPL32* and *YWHAZ* were evaluated, of which *PPIA* and *RPL32* displayed the most stable gene expression (M value = 0.345) and were used in further gene expression analysis of blood samples.

The relative gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (Livak & Schmittgen, 2001), using a geometric average of several reference genes (Vandesompele *et al.*, 2002). The $\Delta\Delta C_q$ is the difference in $C_q$ for a gene of interest between a sample and a calibrator sample, corrected for the $C_q$ difference of the reference gene(s). The formula $2^{-\Delta\Delta Cq}$ provides the fold change (FC) in expression between the two samples, assuming equal efficiency of gene of interest and reference genes in the qPCR reactions.

### 3.3.6 Gene expression profiling using microarray

Microarray analysis with the Affymetrix GeneChip Porcine Genome Array was performed at the Uppsala Array Platform (Paper I, II) according to the manufacturer's instructions. To find DEGs between groups, the empirical Bayes moderated t-test was applied using the *limma* package (Smyth, 2005). This method utilizes the variance from the whole array for each gene, which overcomes the problem of small sample sizes. Multiple testing was corrected for using the method of Benjamini and Hochberg (1995), which provide a *false discovery rate* (*q*-value) for each gene. The false discovery rate method gives an estimation of the number of false positives among the genes detected as differentially expressed on the microarray. This is preferred to traditional multiple testing methods that calculate an adjusted *p*-value based on the risk of finding a single false positive among all genes, which may be too conservative to find any DEGs (Allison *et al.*, 2006). Principal component analysis of the gene expression was also performed to provide a graphical overview of similarity in expression between samples.

Functional enrichment analysis based on DEGs was performed with the web-based program DAVID using gene lists from GO and KEGG (Paper I, II). GO terms were also used to identify genes related to specific functions, especially cytokines and cytokine binding (Paper II). No suitable GO terms were available for some functions, so gene lists from the literature was used to identify PRRs (Lee & Kim, 2007), IRGs (Jenner & Young, 2005) and conserved gene signatures of leukocyte subpopulations (Robbins *et al.*, 2008). GSEA was used for enrichment analysis in cases where manually selected gene lists were required (Paper II), as this was not possible in DAVID. GSEA uses a completely different computational approach, taking into account the relative expression of all the genes on the array when calculation enrichment of genes. However, GSEA and the method used by DAVID typically provide similar results (Huang da *et al.*, 2009). In Paper I, cluster analysis was performed and visualized with a heatmap to identify genes with similar or diverging expression between the two PCV2 isolates. Clustering is useful when comparing multiple experimental groups or samples against each other.

However, the results from muscle and draining lymph node in response to Matrix-M were too diverging to produce meaningful clustering (Paper II).

Validation of findings from microarray results is often made by re-analysing the same sample with a more sensitive method, commonly qPCR. The value of this type of validation may be limited, since a systematic bias is required to affect identification of DEGs (Allison *et al.*, 2006). However, the estimated relative expression might differ as microarrays are semi-quantitative (Arikawa *et al.*, 2008). Further, microarrays and qPCR are not equivalent and may detect different transcript variants when analysing the same gene (Tuggle *et al.*, 2007). Thus, results obtained by microarray and qPCR may not correlate, even though neither is incorrect. No validation was performed for microarray on PCV2-infected intestine (Paper I), whereas a number of genes detected by microarray in response to Matrix-M were analysed by qPCR (Paper II).

# 4 Results and discussion

## 4.1 Clinical, haematological and histological effects of Matrix-M (Paper II, III, IV)

The saponin adjuvant formulation Matrix-M is known to have a good safety and tolerability in humans (Cox *et al.*, 2011). Still, a local reaction at the site of injection is to be expected, as well as reactions in the local draining lymph node where the adaptive immune response develops. Reactions to Matrix-M and related formulations have been evaluated extensively in mice, but there are few reports of early innate immune responses in larger animals that may better reflect those in humans. Therefore, the tolerability and early inflammatory response to Matrix formulations were evaluated in the pig.

Matrix-Q made from non-fractionated Quil-A is considered more reactogenic than the Matrix-M used in experimental human vaccines. The tolerability of subcutaneously injected Matrix-Q at concentrations of 75, 100 and 150 μg was evaluated in one-week old piglets (n = 3) followed for 30 hours (Paper III). No local adverse reactions or decrease in activity was detected in any of the pigs, but one pig given the lowest dose had a low-grade fever 30 hours after administration. Nor did Matrix-M at a intramuscular injection of 150 μg provoke any adverse effects in pigs aged 9 to 11 weeks, when followed for 24 hours (Paper II) or 6 days (Paper IV). The Matrix-M dose of 150 μg was three times higher than the dose used in human clinical trials, for which both good humoral (Cox *et al.*, 2011) and cellular (Pedersen *et al.*, 2014) immune responses were attained when combined with antigen. Both Matrix-M and Matrix-Q should thus be regarded as well tolerated by pigs.

Resident immune cells, such as macrophages and DCs, are rare in muscle tissue during physiological conditions and recruitment of leukocytes to the injection site is essential for development of protection to antigens (Liang & Lore, 2016). Dissection of the injection site one day after intramuscular

administration of Matrix-M showed redness in the injected muscle, indicating a local reaction (Paper II). Histologically, all Matrix-M injected pigs displayed various degrees of local inflammation that was not recorded in any of the control pigs injected with saline. The inflammation was mainly characterized by infiltration of neutrophils, but also lymphocytes and macrophages were present. ISCOMs (Watson *et al.*, 1989) and ISCOMATRIX (Wilson *et al.*, 2012) promote recruitment of immune competent cells to the site of administration. Also the particulate adjuvants alum and MF59 increase the numbers of multiple cell types in the muscle within a day after administration, of which neutrophils are the first to be recruited (Calabro *et al.*, 2011). In contrast, the TLR agonist adjuvants resiquimod and CpG promoted little or no recruitment of cells to injected muscle in mice (Caproni *et al.*, 2012), placing Matrix-M more in line with other particulate adjuvants.

Transport of antigen to the draining lymph node is crucial for induction of an adaptive immune response, but Matrix-M injection induced a prominent reaction of the draining lymph node in pigs also in the absence of any co-administered antigen. Three out of six pigs had enlarged lymph nodes 24 hours after administration, compared to one out of six pigs that received saline (Paper II). Histological examinations revealed a reactive lymphoid hyperplasia in four of the six Matrix-M pigs, which was absent in pigs given saline. Even six days after administration, a reaction in the draining lymph node was detected at gross pathology in four out of eight pigs that had received Matrix-M (Paper IV). In mice, granulocyte numbers increased considerably in the draining lymph node after subcutaneous injection with both Matrix-M (Reimer *et al.*, 2012) and ISCOMATRIX (Duewell *et al.*, 2011). In pigs, histological examination of the draining lymph nodes suggested an influx of eosinophils 24 hours after Matrix-M administration (Paper II), as previously described in mice after injection with MF59 (Calabro *et al.*, 2011). In mice, also DCs, B and T cells were reported to increase in draining lymph node after Matrix-M administration (Reimer *et al.*, 2012), but no such characterizations were made in the present thesis.

Matrix-M seem to be well tolerated by pigs at doses used clinically in vaccines. Intramuscular injections with Matrix-M promoted cell recruitment both to the injection site and draining lymph nodes without showing any clinical signs of illness or discomfort following injection. Thus, the recorded cell migration indicated that Matrix-M elicit a mild inflammation with production of immune mediators that promote recruitment, activation and differentiation of effector cell populations.

## 4.2 Gene expression profiling of innate immune responses in pigs (Paper I, II, IV)

The host response to pathogens and pathogen-derived molecules encompass transcription of many more genes than traditionally measured in immunological studies, and each stimuli may produce different expression profiles (Jenner & Young, 2005). To get an unbiased characterization of the response to Matrix-M, the Affymetrix GeneChip Porcine Genome Array was applied (Paper II). Archived material from PCV2-infected pigs was used to establish necessary methods for this application (Paper I). The focus was to generate RNA of sufficient quality from relevant tissues for this type of analysis and to learn how to extract relevant knowledge from the data. Furthermore, qPCR methods were elaborated to confirm and refine results from the GeneChip array. In addition, a porcine 92-gene qPCR plate array was used to profile the kinetic of transcriptomic responses in blood for five days following Matrix-M injection (Paper IV).

The transcriptional response in the intestine was measured in pigs co-infected with either of two PCV2 isolates (S-PCV2 or PCV2-1010) and PPV, compared to PPV only or to uninfected controls (n = 3; Paper I). Of the 23,256 probesets on the GeneChip array, 14,411 detected expression in pigs from all four groups. Principal component analysis revealed that the response in one pig from each infected group deviated considerably from the other two and had to be excluded in all subsequent analyses, except the GO term enrichment analysis. Biological variation, possibly due to the late sampling time at 28 days post-infection, also prohibited the use of a cut-off for DEGs based on false discovery rate and instead the *p*-value was used together with FC (FC > 2; $p < 0.01$). At that time PCV2-associated disease may appear but with a great inter-individual variation and a low statistical and biological significance (FC > 1.5; $p < 0.05$) was used as cut-off for DEGs in the response to PCV2 in the draining lymph node 21 days after infection (Lee *et al.*, 2010). When selecting DEGs, a criterion based on a combination of FC and q-value is preferred (Allison *et al.*, 2006), but FC alone may be used if the goal is to find genes for functional enrichment analysis (Shi *et al.*, 2008).

Based on gene annotations from Tsai *et al.* (2006) and GO terms for functional annotation, enrichment analysis of DEGs revealed the process *immune response* to be significantly up-regulated in both PCV2 groups, and *inflammatory response*, *defense response* and *complement activation* in the S-PCV2/PPV group. Cluster analysis visualized by a heatmap (Fig. 2 in Paper I) revealed a much larger number of down-regulated genes in the S-PCV2/PPV group than in the PCV2-1010/PPV group. Many of these genes are involved in metabolism and regulation of catabolic processes, which may

reflect the tendency of a more severe pathology, recorded in the S-PCV2/PPV infected pigs. On the other hand, both PCV2-infected groups shared *IFITM3* as the most up-regulated gene. This IRG and antiviral gene (Everitt *et al.*, 2012) had a FC 5 to 7 times higher than the second most up-regulated gene in either group. Despite induction of several other IRGs, *IFITM3* was not reported as up-regulated in intestinal lymph nodes in a time-course study on PCV2 (Tomas *et al.*, 2010). *IFITM3* may therefore be identified as a gene unique for the intestine. Thus, insight into the response to PCV2 was gained despite a biological variation that limited the evaluation of data. It was concluded that the GeneChip array was useful to study transcriptional host responses in porcine tissues.

The transcriptional response to Matrix-M was analysed 24 hours after intramuscular injection and a total of 17,611 and 18,666 probesets detected expression in samples from the injection site and the draining lymph node, respectively. Differentially expressed genes for Matrix-M compared to saline were identified using a cut-off based on both FC and false discovery rate (FC > 2; $q < 0.05$; n = 3; Paper II). By correcting for redundant probesets, 546 genes were found differentially regulated in the injected muscle and 309 genes in the draining lymph node. Injection with the adjuvants MF59, alum or CpG in murine muscle have previously identified a number of "adjuvant core response genes" common for all three adjuvants at the injection site (Mosca *et al.*, 2008). On the porcine GeneChip array, 61 homologues to the "adjuvant core response genes" were present and 20 of these were differentially expressed by Matrix-M. Enrichment analysis on the Matrix-M response revealed 38 GO terms to be enriched in the muscle and 4 in the draining lymph node of which the terms *immune response* and *defense response* were among the top three enriched in both groups.

To study the response in blood over a period of five days in pigs injected with Matrix-M, a custom 92-gene qPCR plate array that focused on innate immunity was used on pooled blood samples (n = 4; Paper IV). About 30% of the genes on the array were up-regulated at any time point in both Matrix-M and saline administered pigs. This clearly reflected the setup of the experiment, where pigs were transported and mixed with non-littermates 18 hours after injection. These stressors are known to affect blood parameters in pigs such as cortisol, granulocytes (Dalin *et al.*, 1993) and acute-phase proteins (Salamano *et al.*, 2008; Pineiro *et al.*, 2007), which also complicated interpretation of the gene expression results. However, a delay of the induction of gene expression was indicated for the saline injected pigs. Comparing the FC values at 18 hours post injection revealed a relative up-regulation (> 2-fold expression) in Matrix-M injected pigs compared to saline for 19 genes. Five of these genes

(*IL18*, *MYD88*, *NLRP3*, *TLR4*, *TLR9*) were subjected to validation on individual samples collected from all pigs in the study at 18-hours after administration (n = 8), but none of the genes were significantly up-regulated. It is possible that the non-normal distribution of gene expression levels (Kubista *et al.*, 2006) skewed the results from the qPCR plate array when pooling samples.

In conclusion, the GeneChip array detected immunological perturbations in pigs in vivo and annotations based on homologous human gene names facilitated the functional enrichment analysis using GO terms. The DEGs and enriched GO terms detected for PCV2 were in accordance with clinical findings but also emphasized the need to reduce the biological variation by careful experimental design. Indeed, the gene expression in response to Matrix-M adjuvant measured 24 hours after administration was rather uniform and identified activation of genes related to the innate immune system in a way that motivated deeper analysis.

## 4.3 Gene expression in SPF pigs after Matrix-M administration (Paper II, III, IV)

A large number of genes were affected locally after Matrix-M administration. The cellular processes at the injection site and in the draining lymph node suggested cytokines and chemokines to be involved in the response. GO terms were used to identify such genes in the microarray data, and a total of 19 genes coding for cytokines or cytokine binding proteins were differentially expressed at the injection site 24 hours after Matrix-M administration (Table 5 in Paper II). In the draining lymph node, 11 such genes were affected. Of several adjuvants evaluated in mice, only the TRL2 agonist $Pam_3CSK_4$ regulated a substantial amount of cytokines both at the injection site and in the draining lymph node (Caproni *et al.*, 2012). Other adjuvants in that study either affected cytokine genes solely at the injection site (MF59, alum, CpG), or predominantly in the draining lymph node (resiquimod). This separates Matrix-M from other particulate adjuvants and indicates a mechanism of action more similar to a direct PRR ligand. However, based on some data (Wilson *et al.*, 2012), it has been argued that Matrix-formulated saponin adjuvants probably do not signal through TLRs (Lövgren-Bengtsson *et al.*, 2013; Morelli *et al.*, 2012).

Chemokine genes up-regulated by Matrix-M at the injection site included *CCL2* that attract monocytes and DCs, and *CXCL2* that recruit neutrophils (Charo & Ransohoff, 2006). This may in part explain the macroscopical and histological findings at the injection site after Matrix-M administration in pigs

(Paper II). *CCL2* has been identified as an "adjuvant core response gene" in mice (Mosca *et al.*, 2008) and was up-regulated in porcine skin after injection either with CpG, Emulsigen or polyphosphazene (Magiri *et al.*, 2016). *CCL2* expression is thus likely to mirror adjuvant responses also in pigs.

The cytokine genes *IL10* and *IL18* were up-regulated by Matrix-M at the injection site (Paper II). In the draining lymph node, *IL1B* was up-regulated, while *IL18* was down-regulated. In blood, expression of *IL1B* and *IL18* was affected by transport and mixing stress, but there was no differences in expression between pigs receiving Matrix-M or saline in advance (Paper IV). Caspase-1 is required for IL-1β and IL-18 release after inflammasome activation (Rathinam & Fitzgerald, 2016), and it is interesting that the *CASP1* gene was also up-regulated by Matrix-M at the injection site (Paper II). Furthermore, the inflammasome-associated receptor gene *NLRP3* tended to be increased in blood from pigs receiving Matrix-M, compared to saline at 18 hours post injection. The NLRP3 inflammasome may be activated in vitro by ISCOMATRIX (Wilson *et al.*, 2014) as well as Matrix-M and the Quil-A saponin QS-21 (Marty-Roix *et al.*, 2016). However, as NLRP3 is not constitutively expressed, pre-treatment with a TLR4-agonist or TNF-α was required for all three adjuvants. Up-regulation of genes for IL-1β, IL-18, caspase-1 and possibly also NLRP3 by Matrix-M can therefore indicate preparedness for inflammasome activation and IL-1β/IL-18 release in vivo.

As combining particulate adjuvants with specific PRR agonists has been suggested recently for future vaccine designs (O'Hagan & Fox, 2015), the capacity of Matrix-M to modulate the expression of PRRs was evaluated. A number of PPR genes were up-regulated also at the injection site after Matrix-M administration, including *TLR2*, *TLR4*, the TLR-associated *MYD88* and *PTX3*, which encodes the soluble PRR pentraxin 3 (Fig. 2 in Paper II). In mouse muscle, pentraxin 3 was up-regulated both at gene level and as protein on muscle cells after injection with MF59 or CpG (Mosca *et al.*, 2008). Pentraxin 3 is expressed on porcine bone marrow-derived DCs and increased in serum following influenza infection in pigs (Crisci *et al.*, 2014). *TLR2* was up-regulated compared to saline in whole blood collected 18 hours after Matrix-M administration (Paper IV). At reanalysis of material from Paper II, up-regulation of *TLR2* in PBMCs collected 17 hours after Matrix-M administration was detected in three out of six pigs (Fig. 2). Both granulocytes and PBMCs from pigs express *TLR2* (Alvarez *et al.*, 2008), so the increase in *TLR2* expression may have been confined to granulocytes that were not included in the analysis of PBMCs. In murine muscle, *TLR2* was unaffected by the adjuvants alum, MF59, CpG, resiquimod and $Pam_3CSK_4$ (Caproni *et al.*, 2012; Mosca *et al.*, 2008) but was significantly up-regulated by the TLR4

agonist adjuvant glucopyranosyl lipid A (Lambert *et al.*, 2012). *TLR2* might be useful as a biomarker for Matrix-M stimulation, but surprisingly the *TLR2* expression was down-regulated by Matrix-M in all cell populations under in vitro conditions (Paper IV).

Taken together, Matrix-M has a pronounced effect on expression of genes related to immune modulation both at the local injection site and in the draining lymph node. Although less pronounced, Matrix-M also induced measurable changes of the transcription in blood. The up-regulation of chemokine genes is presumably related to the influx of immune cells to the injection site and the draining lymph node and this transition of cells at various stages of activation might explain why early adjuvant effects also have been studied with some success in the blood (Pulendran *et al.*, 2010). Matrix-M also modulate the expression of genes for PRRs and associated mediators (TLR2, TLR4, MyD88 and cytosolic RNA sensors) and inflammasome-associated genes (*IL1B*, *IL18*, *CASP1*). These results give an insight into possible mechanisms exerted by Matrix-M in priming the innate immunity of the host.

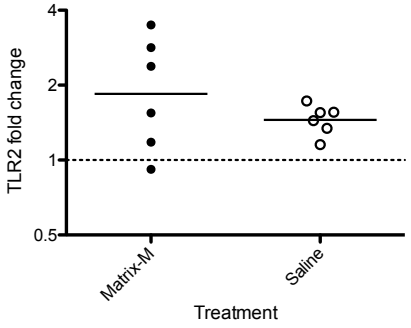


*Figure 2.* Expression of *TLR2* in PBMCs from porcine blood collected 17 hours after intramuscular injection with Matrix-M or saline. Expression was reanalysed with qPCR from archived material (Paper II) according to methods in Paper IV. The fold change was calculated against the expression in PBMCs 24 hours before injections. Individual fold change and geometric mean.

## 4.4 Profiling of interferon-related response after Matrix-M administration (Paper II, III, IV)

Several of the most up-regulated genes in the response to Matrix-M at the injection site and in the draining lymph node were IRGs, which sparked the question if this was a true specific response or just a random effect due to the sheer number of IRGs in the genome. Gene set enrichment analysis of the transcriptional response to Matrix-M identified IRGs to be highly enriched ($q < 0.001$) both at the injection site and in the draining lymph node (Paper II). IRGs constituted 38 out of 384 (10%) up-regulated genes at the injection site and 40 out of 92 (43%) up-regulated genes in the draining lymph node. The IRG response differed vastly between the tissues as only two IRGs were commonly up-regulated (Fig. 3 in Paper III) which was consistent with

previous reports on differences in gene expression between injection site and draining lymph node in response to adjuvants (Caproni *et al.*, 2012; Lambert *et al.*, 2012). Involvement of a type I IFN response was further corroborated by enrichment of a gene signature for pDCs ($q < 0.05$) at the injection site (Paper II).

IFN genes present and annotated on the GeneChip array were *IFNA2*, *IFNA6*, *IFNA8*, *IFNB1* (IFN-β) and *IFNG* (IFN-γ). For both tissues, none of these genes were differentially expressed and the expression of *IFNB1* was below background. The transcription of *IFNG* tended to be up-regulated in the draining lymph node (FC = 4.5; $q = 0.087$). This lack of IFN gene induction could be due to the kinetics of the type I IFN response, as IFN genes are typically transiently expressed and then promote the subsequent induction of IRGs (Jenner & Young, 2005). It is also possible that IFN-α genes not present on the array were a part of the transcriptional type I IFN response. In a similar manner, injection of CpG in porcine skin down-regulated IFN-α gene expression at all time points for up to four days, concurrent with a high up-regulation of several IRGs (Magiri *et al.*, 2016). Nevertheless, gene expression analysis with qPCR for the draining lymph node revealed a prominent induction of IFN-β transcription in four out of six Matrix-M administered pigs ($p < 0.05$; Paper III) but revealed no regulation of IFN-α. The IRGs *IRF7*, *MX1* and *OAS1* were up-regulated in the draining lymph node (Paper II). Screening using the qPCR plate array in blood indicated *IRF7* as up-regulated 18 hours after Matrix-M injection, but none of the genes for IFN-α, IFN-β and IFN-γ nor the IRGs *MX1* and *OAS1* were affected (Paper IV). However, qPCR analysis on individual pigs presented a small but significant up-regulation of IFN-α (relative FC = 1.83; $p < 0.05$) in blood 18 hours after Matrix-M administration (Paper IV).

A type I IFN response has not been described previously for ISCOM-Matrix formulations. Such responses are typically associated with detection of nucleic acids, through TLRs, cytosolic RNA sensors or DNA sensing via the adaptor protein STING. However, a nucleic acid-independent induction of type I IFNs through STING by cell membrane fusion with liposomes or virosomes has been described (Holm *et al.*, 2012). The saponins in ISCOM-Matrix engage cell membranes and may speculatively function in a similar way. The type I IFN-associated transcriptional response induced by Matrix-M included both IFN genes and IRGs, and could be detected both at the injection site, in the draining lymph node and in blood (Paper II, III, IV). IFN-α is known to increase CTL responses by promoting cross-presentation (Le Bon *et al.*, 2003). Indeed, the levels of late T cell responses to vaccination in mice correlated with early expression in blood of the IRGs *MYD88*, *STAT1* and *DUSP5*

(Derian *et al.*, 2016) and *IRF7* was one of the key transcription factors up-regulated in response to an effective human Yellow fever vaccine (Gaucher *et al.*, 2008). All these IRGs were induced by Matrix-M in the draining lymph node, which may contribute to the CTL responses typically induced by Matrix-formulated saponin adjuvants.

## 4.5 In vitro exposure of blood cells to Matrix-M (Paper III, IV)

Matrix-M promotes a prominent influx of neutrophils, induces a type I IFN-related transcriptional response and a gene signature of pDCs at the injection site. In patients with systemic lupus erythematosus, autologous DNA in NETs from neutrophils is bound to autoantibodies and taken up by pDC via Fc-receptor mediated endocytosis, thereby promoting IFN-α production (Garcia-Romo *et al.*, 2011; Lande *et al.*, 2011). Porcine pDCs may also respond to self-DNA in a similar fashion (Baumann *et al.*, 2014). Thus, the capacity of Matrix-M to promote NET formation was evaluated. Stimulation with the positive control PMA induced NET formation in porcine polymorphonuclear leukocytes after four hours (Fig. 3 in Paper III). NETs were manifested as cells with condensed nucleus and genomic content released in fibre-like structures, as previously described for humans (Brinkmann *et al.*, 2004) and pigs (Scapinello *et al.*, 2011). In contrast, Matrix-M in concentrations from 0.3 to 3 μg/ml did not induce NETs, not even after 16 hours stimulation time (Paper III). Yet, exposure to Matrix-M for 16 hours promoted both condensation of nuclei, disintegration of cells and formation of multiple DNA-containing fragments around the cells, indicative of pyroptosis (Labbé & Saleh, 2011). Pyroptosis was further supported by up-regulation of *CASP1*, *IL1B* and *IL18* by Matrix-M in vivo (Paper II) and the inflammasome activation reported for Matrix-M (Marty-Roix *et al.*, 2016) and ISCOMATRIX (Wilson *et al.*, 2014). In contrast, no apoptosis or necrosis was detected in PBMCs after 18 hours of in vitro exposure to Matrix-M, using equal concentrations as for the neutrophils (Paper III).

The transcriptional response to Matrix-M was evaluated in vitro to distinguish influence of different cell types. A slight up-regulation of the gene for TNF-α was detected in PBMCs exposed to Matrix-M for six hours in vitro, (Paper III), but not genes for other cytokines readily induced by LPS or CpG (IFN-α, IFN-γ, IL-1β, IL-6, IL-10, IL-12p40, transforming growth factor [TGF]-β). PBMCs depleted of adherent cells, cultured for 16 hours and exposed to Matrix-M for 6 hours did not change their expression of any gene analysed (Paper IV). ISCOMATRIX induced barely any cytokines in murine macrophages and DCs generated in vitro (Wilson *et al.*, 2012) and alum and

MF59 did not induce any cytokine genes in splenocytes in vitro, in contrast to stimulation with $Pam_3CSK_4$, resiquimod and CpG (Caproni *et al.*, 2012). However, both alum and MF59 up-regulated CXCL8, CCL4 and IL-1ra in human monocytes and monocyte-derived macrophages, but not in PBMCs or monocyte-depleted PBMCs (Seubert *et al.*, 2008). The response to Matrix-M in monocytes following a 6-hour exposure included up-regulated genes for CXCL8 and for IL-1β after overnight culture (Paper IV). Both CXCL8 and IL-1β were increased in efferent lymph in sheep after ISCOMATRIX administration (Windon *et al.*, 2000). Porcine lymphocytes cultured and exposed to Matrix-M for three days up-regulated genes for the pro-inflammatory cytokines IL-1β and CXCL8 and the $T_H$ associated cytokines IL-12p40, IL-17A and IFN-γ, while genes for the immunoregulatory cytokines IL-10 and TGF-β were down-regulated (Paper IV). The same lymphocyte cultures exposed to Matrix-M only for the last six hours had a similar but less pronounced profile. Modulation of gene expression for IL-12p40, IFN-γ and IL-10 might explain the $T_H1$-associated responses seen for Matrix-M vaccines (Pedersen *et al.*, 2014; Magnusson *et al.*, 2013; Madhun *et al.*, 2009). In contrast, *IL10* was up-regulated at the injection site 24 h after intramuscular administration (Paper II), possibly dampening the inflammation at that time.

MoDCs exposed to Matrix-M for six hours did not change the expression of any cytokine gene analysed, except for a small increase in the expression of IFN-α. Presence of Matrix-M during the full 5-day generation of MoDCs however, promoted an increase in IFN-α expression and also induced substantial levels of IL-6 transcripts. Thus, a type I IFN response was indicated transcriptomically in vitro, as was detected both at the injection site, in the draining lymph node and in blood. However, expression of IFN-β, or the IRGs *IFITM3*, *SPP1* and *STING* was not significantly affected in any of the cell cultures.

Similar to other particulate adjuvants, Matrix-M induced little or no expression of cytokine genes when examined in vitro cell populations. Specifically, the in vitro system was not sufficient to reproduce the potent induction of type I IFN-related genes detected in vivo. DAMPs are released into the local tissue after injection with alum (Marichal *et al.*, 2011) and MF59 (Vono *et al.*, 2013) that contribute to their adjuvant effect. The increased responses after prolonged exposure to Matrix-M likely caused release of DAMPs, which suggests involvement of DAMPs. Together with the fact that muscle cells may also be involved in the early immune response to adjuvant injection (Liang & Lore, 2016), simultaneous cultures of multiple cell types or ex vivo tissue explants could be more effective to study particulate adjuvants including Matrix-M.

## 4.6 Effects of Matrix-M in a contact exposure model (Paper IV)

The immunomodulatory effects of Matrix-M suggest that it could be useful in emergency vaccines, where the aim is to delay infection by innate immune stimulation until adaptive responses appear (Foster *et al.*, 2012). The induction of a type I IFN response by Matrix-M is especially interesting considering the antiviral effects, and IFN-α has previously been used with the aim to prevent PRRSV in pigs (Brockmeier *et al.*, 2009). To investigate if Matrix-M could be used clinically to dampen infection, a contact exposure model was set up where SPF pigs were administered Matrix-M or saline and mixed with conventionally reared pigs (Paper IV). The pigs were also subjected to transport and mixing stress to provoke replication of for example PCV2 and/or *H. parasuis* that had been demonstrated in pigs from the SPF herd. The transport and mixing was followed by an increase in granulocyte counts and SAA levels for all SPF pigs, in accordance with earlier reports (Salamano *et al.*, 2008; Pineiro *et al.*, 2007; Dalin *et al.*, 1993). Also the expression of *IL1B*, *IL18*, *MYD88*, *NLRP3*, *TLR2* and *TLR4* was transiently increased in blood after transport and mixing but no effects of Matrix-M on these parameters were discerned.

All SPF pigs mixed with conventional pigs developed respiratory disease in the contact exposure model (Paper IV), which was confirmed in seven out of eight pigs at necropsy. The conventional pigs remained healthy throughout the study, but lung lesions recorded at necropsy for these pigs revealed evidences for respiratory diseases. No signs of respiratory disease were recorded in any of the SPF pigs that were mixed with other SPF pigs (control), or in any SPF pig before the contact exposure. Two pigs in the group receiving saline displayed decreased general condition during the study and became lame, confirmed by joint lesions at necropsy. These symptoms were consistent with Glässer's disease induced by *H. parasuis* infection (Oliveira & Pijoan, 2004), but *H. parasuis* was not demonstrated at necropsy. These two pigs had increased levels of granulocyte counts and SAA levels throughout the experiment, and SAA levels are known to correlate well with clinical symptoms and disease severity (Sjolund *et al.*, 2011; Cray *et al.*, 2009; Hulten *et al.*, 2003). Similarly, these pigs had increased gene expression of *IL18* and *TLR2* throughout the experiment, in contrast to the other pigs. No signs of systemic disease were detected clinically or at necropsy in any other SPF pig, including all four pigs that received Matrix-M.

All blood parameters measured declined to baseline after the initial transport and mixing stress response in the pigs administered Matrix-M. However, in three out of these four pigs, there was an increase in granulocyte counts, SAA levels and gene expression for *IL1B*, *IL18*, *MYD88*, *TLR2* and *TLR4* on day 5 or 6. Despite lack of clinical symptoms, it is likely that this

reflected the beginning of systemic disease also in the Matrix-M pigs. A similar kinetic was seen for pigs in a contact exposure experiment with PRRSV (Li *et al.*, 2013), where vaccination alleviated and delayed symptoms for up to five days after inoculation with PRRSV in contact pigs on the same day, compared to unvaccinated controls. Similarly, Matrix-M may have delayed or diminished systemic disease development at contact exposure (Paper IV).

The contact exposure model simulated mixing of pigs with various health statuses, mimicking field conditions at allocation of grower pigs, and successfully provoked respiratory disease in all exposed SPF pigs. Symptoms that resembled Glässer's disease, and correlated with SAA levels, granulocyte counts and the expression of several genes analysed, were only detected in two SPF pigs receiving saline and not in pigs given Matrix-M. Despite the limited number of animals, this indicated that Matrix-M modulated the disease kinetic in pigs following transport, mixing and exposure to new pathogens.

# 5 Conclusions

From results in this thesis it can be concluded that:

- The Affymetrix GeneChip Porcine Genome Array can be successfully used to study responses to various immunological stimuli in porcine tissues.
- Matrix-M is well tolerated by pigs. It promotes a mild local inflammation both at the injection site and in the draining lymph node, causing a redistribution of immune cells as evidenced by an altered expression of genes encoding chemokines and chemokine receptors.
- Matrix-M affects gene expression of cytokines, pattern recognition receptors and associated proteins and thereby primes cells for further immune regulation.
- Based on circumstantial evidence, Matrix-M induces a type I interferon response in pigs.
- In vitro responses to Matrix-M differ from those in vivo and show a considerable variation depending on cell type and culture condition.
- A contact exposure model in pigs was successfully established to reproduce respiratory disease in grower SPF pigs.
- Based on a limited number of pigs, administration of Matrix-M in the contact exposure model could delay the onset of systemic disease.

# 6 Future perspectives

The gene expression profiling provided valuable information on the response to Matrix-M in vivo not readily detected using traditional methods. Microarray or RNA-Seq technology can be used in the pig to illuminate differences between various Matrix formulations and/or saponin-based adjuvants. As combinations of Matrix formulations and immunomodulatory molecules have been used with success in murine vaccines, the early gene signatures of such combinations are also worth exploring. Characterization of the early response to Matrix formulations or other adjuvants together with antigen can help finding correlates with long-term immunity in a systems biology approach. With the possibility of porcine infection models, gene expression profiling should enable the finding of innate immune signatures for adjuvants correlating with protection, highly sought for in vaccine development research.

Whole tissue or blood gene expression profiling provide limited information on the cellular origin of the response. Phenotypic characterization of the cells involved in the early response to saponin-based adjuvants such as Matrix-M would help understanding their mode of action. Archived material preserved in liquid nitrogen or as formalin-fixed, paraffin-embedded tissues are available for such studies.

The interferon response induced by Matrix-M is puzzling as no available model fully explains it. Merging of cell membranes can induce interferon in the lack of nucleic acid or other direct PRR ligands, but do not explain the weak type I interferon responses detected in vitro. ISCOM-Matrix can promote escape of antigen to the cytosol that may also occur for DAMPs present or released at injection. Novel in vitro systems should therefore include defined DAMP molecules (nucleic acid, heat-shock proteins, free ATP, etc.) when exposing cells to Matrix formulations. To imitate the in vivo situation, less defined sources of DAMPs may be attained by using tissue explants or co-cultures of tissue cells and immune cells.

The results presented for Matrix-M suggests using it clinically as an immunomodulator or in emergency vaccines to combat infection by means of innate immune activation. In addition to periods of stress, such as transport and mixing of groups with different health statuses, there are porcine diseases for which there are few or no effective vaccines available. Field studies using Matrix formulations clinically as immunomodulators or in emergency vaccines are therefore motivated.

# 7 Populärvetenskaplig sammanfattning

Vaccinering är ett av de bästa sätten att skydda en värd mot infektion, genom att skapa långvarig immunitet mot en patogen (sjukdomsalstrande mikroorganism). Ett vaccin består av en patogen-specifik del som immunförsvaret känner igen och bildar skydd emot, samt en immunstimulerande del som drar igång immunsvaret. De immunstimulerande delarna består av varningssignaler som kan vara kroppsfrämmande, ofta ämnen från patogener, eller kroppsegna signalämnen från skadade eller döda celler. Varningssignaler känns igen av kroppens immunceller genom speciella varningssignalreceptorer. Traditionella vaccin av döda eller försvagade patogener innehåller naturliga varningssignaler, men moderna vaccin kräver ofta hjälp av så kallade adjuvans som verkar genom att aktivera dessa varningssignalreceptorer. Eftersom adjuvans aktiverar immunreaktioner kan de även användas för att tillfälligt bekämpa en infektion, vilket finns ett behov av inom modern djurproduktion.

En typ av adjuvans är framrenade saponiner ur barken från såpträdet (*Quillaja saponaria)*. Om man blandar *Quillaja*-saponiner med ämnen liknande de som finns i cellmembran så bildas en sorts nanopartiklar. Matrix-M är ett sådant adjuvans som för närvarande undersöks i kliniska studier för humana vaccin, men liknande adjuvans har under lång tid använts i veterinära vaccin. Hur dessa adjuvans fungerar är till stor del fortfarande okänt, vilket skapar svårigheter om man vill förbättra dem på ett förutsägbart sätt. Denna avhandling har undersökt de tidiga immunreaktionerna mot Matrix-M hos grisar, både i det levande djuret och i blodceller samlade från grisar och stimulerade på labb. Studierna på Matrix-M gjordes utan patogen-specifika vaccindelar för att undersöka den särskilda effekten av adjuvanset.

När kroppen reagerar på ett immunologiskt stimuli, oavsett om det är en patogen eller ett adjuvans, så förändras uttrycket av ett stort antal gener. Med så kallad *microarray*-teknik kan man mäta uttrycket av samtliga gener som finns hos en individ, för däggdjur ungefär 20 000 stycken. Med hjälp av

datorbaserade analyser kan man skapa en profil utifrån vilken typ av stimuli som påverkat individen. I början av detta arbete upprättades en metod för att använda microarray för att mäta immunreaktioner i grisvävnad genom att undersöka arkiverat material från virusinfekterade tarmar.

Injektion med Matrix-M i grisar orsakade ett tydligt inflöde av immunceller till den injicerade muskeln, men även till den närliggande lymfknutan. Detta är intressant eftersom det framför allt är i lymfknutan det långvariga immunsvaret bildas. Microarray-analyserna påvisade ökat genuttryck för så kallade cytokiner och kemokiner i båda dessa vävnader, proteiner som rekryterar och aktiverar immunceller. Även gener för varningssignalreceptorer ökade sitt utryck, vilket tillsammans tyder på att Matrix-M kan skapa en aktiv immunologisk miljö efter injektion som troligen är gynnsam vid vaccinering. Genuttrycket för en av dessa varningssignalreceptorer (TLR2) var även förhöjt i blod, och denna skulle kunna användas som biomarkör för den tidiga effekten av adjuvans. Matrix-M aktiverade även gener för så kallade interferoner och interferon-relaterade gener, signalämnen aktiva mot virusinfektion men som vid vaccination även främjar ett långvarigt skydd mot framför allt virus. Interferon-svaret kunde detekteras både i vävnad och blod från injicerade grisar, och i viss mån i blodceller som odlats på labb. Generellt var dock immunreaktionerna mot Matrix-M i odlade blodceller svaga i förhållande till vad som sågs i den levande grisen. Mer arbete krävs för att hitta bra system där man på labb kan undersöka effekten av denna typ av adjuvans.

Den immunstimulerande effekten av Matrix-M undersöktes till sist i en infektionsmodell. Smittfria grisar, som till stor del saknar skydd mot vanliga patogener som drabbar gris, behandlades med Matrix-M eller koksaltlösning (kontroll) innan de transporterades och blandades med konventionellt uppfödda grisar. Denna stress och utsatthet för ny smitta framkallade luftvägssjukdom i samtliga smittfria grisar. Några av de som inte fått Matrix-M visade dessutom tecken på transportsjuka, en infektionssjukdom som kan ge påverkat allmäntillstånd och ledproblem. Dessa symptom sågs samtidigt som förändringar i blodbilden och förhöjning av inflammations-markören SAA och genuttryck i blodet. Behandling med Matrix-M verkade alltså kunna hämma utvecklingen av dessa symptom.

Sammantaget visade detta projekt att microarray är en fungerande metod för att utvärdera adjuvans. Matrix-M aktiverar en tydlig immunreaktion i grisar som troligen är gynnsam när man inkluderar Matrix-M i vaccin. Immunreaktionerna orsakade av Matrix-M skulle även kunna användas som förebyggande behandling i djurhållning vid skeden då det finns risk för infektion framkallad av stress, som transporter eller när man blandar djur från olika grupper.

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